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Tissue culture and transformation of introducing genes useful for pest management in rice

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**TISSUE CULTURE AND TRANSFORMATION FOR INTRODUCING GENES
USEFUL FOR PEST MANAGEMENT IN RICE**

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

By
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ABSTRACT

Sheath blight (SB), caused by *Rhizoctonia solani* Kühn, is a major rice disease internationally and in the southern rice area of the United States, including Louisiana. Breeders have incorporated partial resistance into commercial rice varieties to control the disease, but a higher level of resistance is needed. It has been demonstrated that the pathogenesis-related (PR) proteins β -1, 3-glucanase and chitinase are components of effective defense mechanisms for protecting plants against fungal pathogens. This research was conducted to co-transform the β -1, 3-glucanase, chitinase and *bar* genes into the rice variety Taipei 309 using the *hpt* gene for resistance to hygromycin B as a selective marker. Transformed calli and regenerated plants were screened with hygromycin B, and the plants were then further tested for resistance to Liberty herbicide and *Rhizoctonia solani*.

Methods were developed to screen transgenic plants for resistance to hygromycin B and Liberty herbicide using dip and cut in toxicant solutions. Five of 99 plants in the field test and 51 of 55 plants in greenhouse test were highly resistant to Liberty herbicide. The tooth-pick inoculation method was used to test transformed plants for SB resistance. Seventeen transgenic plants in the field test and 10 transgenic plants from greenhouse tests were highly resistant to SB. Fourteen of the 17 SB resistant plants were also resistant to hygromycin B, one of the plants was highly resistant to Liberty herbicide, and 9 of the 17 SB resistant plants had moderate resistance to Liberty.

Panicle blight, caused by *Burkholderia glumae*, has been an important bacterial disease in rice worldwide and in Louisiana. No effective pesticides are available to control this disease. The PR protein thionin is reported to control certain bacterial diseases in plants. In this study, the thionin production, *bar*, and *hpt* genes were co-transformed to the rice variety Lafitte. Resistance

to hygromycin B, Liberty herbicide, *Xanthomonas oryza* and *B. glumae* were expressed in selected transformed Lafitte plants.

This research has created, through transformation, new sources of resistance to two major rice pathogens that cause major losses to rice. These resistances can be transferred to commercial varieties by conventional breeding methods.

CHAPTER 1

REVIEW OF LITERATURE

Plant disease control is a major challenge to agriculture worldwide due to significant yield losses in crops caused by plant diseases. The concept of “integrated pest management”, or IPM, has led to the development of useful pest management measures. Pest control measures throughout the world cost billions of dollars each year. However, use of pesticides is becoming more problematic due to development of resistance and to environmental concerns.

Conventional plant breeding has made significant impact by improving the resistance of many crops to important diseases, but the time-consuming processes of making crosses, back crosses, and progeny selection makes it difficult to react quickly to the evolution of new virulent pathogen races. Moreover, plant breeding techniques are not a solution to many major diseases because there are no natural sources of resistance available to the breeder (Dasgupta, 1992; Melchers and Stuiver, 2000).

Plant genetic engineering has been used to transfer alien genes to plants and thereby produce plants resistance to bacterial or fungal diseases through expression of the introduced genes (Clausen et al. 2000; Datta et al. 1999, 2000, 2001; Iwai et al. 2002; Narayanan et al. 2002; Tabei 1998; Tang et al., 1999).

1.1 RICE SHEATH BLIGHT DISEASE

Rice sheath blight disease (SB), caused by the fungus *Rhizoctonia solani* Kühn, is considered to be an internationally important disease of rice (*Oryza sativa* L.), which is second among fungal diseases only to rice blast in causing yield loss (Lee and Rush, 1983; Ou, 1985). Sheath blight is one of the most important rice diseases worldwide over the past 25 years and has been the most economically significant disease in Louisiana rice since the early 1970's (Lee and

Rush, 1983; Xie et al., 1990; 1992). Breeders have used traditional breeding methods to select moderate levels of partial resistant in cultivars to control SB (Rush et al. 1995, 1996; Xie et al., 1992), but higher levels of resistance are needed as no source of complete resistance is known for SB.

1.1.1. Sheath Blight Development and Yield Loss

R. solani causes large ovoid spots on leaf sheaths and irregular spots on leaf blades. The lesions have grayish-white or light green centers with a brown or reddish brown margin, and as lesions coalesce on the sheath, the blades develop a yellow-orange color and eventually die (Groth et al. 1991). At the boot stage of growth, the disease on lower leaf sheaths develops more rapidly, and at the heading stage, disease on upper leaf sheaths develops very rapidly. Sclerotia are produced on healthy tissues near lesions and detach from the plant and fall to the soil at maturity. Sclerotia can survive in the soil between crops, and along with the fungus in plant debris from the previous crop, serve as primary inoculum (Lee and Rush, 1983).

Environment factors are very important in SB development. The optimum temperatures for disease development range from 30 to 32C and a high relative humidity of 96-97% is critical for disease development (Hashiba, 1985; Shi and Cheng, 1995).

Rice growth stage is also an important factor for SB development and yield loss. Damage due to SB may occur at any stage, but yield loss is higher when infection occurs at the booting or flowering stages of growth (Sharma et al., 1990; Vanitha et al., 1996). When SB lesions extended to the flag leaf, yield loss can be as high as 25% and a 30-40% yield loss can occur with severe infection of leaf sheaths and blades (Kozaka, 1970).

1.1.2 Sheath Blight Control

It is very difficult to control SB using cultural practices. Chemicals used for SB control, such as Quadris® (common name: azoxystrobin, manufactured by Syngenta Crop Protection Canada Inc.) and Moncut® (common name: flutolanil, manufactured by Gowan Company) are effective, but pesticide use is expensive and may cause environmental concerns (Lee and Rush, 1983; Groth et al. 1993; Sha, 1998). Host resistance is the most desirable approach to SB control, but no complete SB resistance has been identified in rice (Sha, 1998). Pan et al. (1995) showed that significant partial resistance is available for SB, and that partial resistance may be controlled by single major genes as well as minor genes. Despite extensive research by pathologists and breeders, it has been difficult to use traditional breeding methods to produce SB resistant rice varieties. Although some progress has been made in using partial resistance genes for SB control (Pan, 1995), the use of transgenes may provide higher and more durable resistance. Plant genetic engineering has been used to transfer foreign genes to rice and different levels of enhancement of sheath blight resistance were demonstrated (Datta et al., 1999, 2000, 2001). However, no high level SB resistant rice cultivars have been developed so far using these procedures.

1.2 RICE PANICLE BLIGHT DISEASE

Bacterial panicle blight disease caused by *Burkholderia glumae* (formerly *Pseudomonas glumae*) has been reported in many countries including Japan (Uematsu et al., 1976b), Taiwan (Chien and Chang, 1987), Philippines (Cottyn et al., 1996a; 1996b), Latin America (Zeigler et al., 1987; Zeigler and Alvarez, 1987), and the United States (Rush et al., 1998). This bacterium causes grain rot on rice and is also responsible for causing bacterial wilt in many field crops (Jeong et al., 2003).

The bacterium was first described in Japan as causing rice brown stripe and grain rot (Uematsu et al, 1976a; 1976b). In Japan, raising rice seedlings has changed from anaerobic conditions, from seeding into water, to the aerobic conditions of upland seedling nurseries or to boxes in sheds where high temperatures and humidity are maintained. However, seedlings grown at high temperatures in upland nurseries or in sheds are susceptible to bacterial pathogens, such as the seed-transmitted bacteria *B. plantarii* and *B. glumae*, which cause seedling blight disease (Iwai et al., 2002). Grain rot and seedling blight caused by *B. glumae* has become a major topic of research in Japan (Iwai et al., 2002).

The cause of panicle blight (PB) in Louisiana was unknown in 1991 when the disease was characterized by brown or straw-colored discoloration of florets on a panicle, the grain stopped developing, the florets turned gray, and panicles remained upright as the grain did not fill (Groth et al. 1991). Panicle blight on rice has been a recurrent problem in Louisiana and other Southern rice production areas for more than 40 years. In 1995 and 1998, panicle blight of rice was prevalent and severe in Louisiana, Arkansas and Texas. The bacterium *B. glumae* was first identified as a causal agent of panicle blighting of rice in Louisiana in 1996 and the disease was called bacterial panicle blight (BPB) and sheath rot (Shahjahan et al. 2000).

1.2.1 Bacterial Panicle Blight Development and Yield Loss

Development of severe BPB disease appears to be associated with unusually hot weather, warm nights and high humidity during the heading stages (Shahjahan et al. 2001). The critical stage for infection is at panicle emergence and flowering. The disease causes floret sterility, kernel abortion, discoloration of the developing grains and significant yield loss. The highly virulent bacterium may cause yield losses as high as 70% in the field (Shahjahan et al. 2001).

Jeong et al. (2003) reported that in Korea, rice grain rot caused by *B. glumae* occurred at the flowering stage, when temperature and moisture were high, and caused yield losses up to 34%.

1.2.2 Bacterial Panicle Blight Control

Chemicals such as antibiotics, copper, and copper-containing compounds have been used in the management of this disease. Hikichi (1993, 1995) found that oxolinic acid had antibacterial activity against *Pseudomonas glumae*. Rush et al. (2001) also evaluated chemicals for controlling the disease and pointed out that oxolinic acid (Starner) was effective against BPB, but the level of disease control has been limited and the bacterium has been reported to acquire resistance to bactericides (Iwai et al., 2002). Screening of rice germplasm for resistance showed that only 1% of 238 entries screened were resistant (Shahjahan 2001). Suitable genetic sources of resistance to *B. glumae* for crossing with Japonica rice cultivars could not be found (Iwai et al., 2002).

Methods to control the disease more effectively have not been found. Transgenic rice plants over-producing an oat cell-wall-bound thionin were reported to have enhanced resistance to bacterial diseases (Iwai et al., 2002), suggesting that plant genetic engineering may provide opportunities to control this disease.

1.3 TRANSFORMATION OF PLANTS

Since the mid-1980s, great progress has been made in transformation technologies (Chibbar et al., 1994; Chen et al., 1998; Gahakwa et al., 2000). Different foreign genes cloned from bacteria and plants have been transferred into major crops such as rice (McElroy et al., 1990; Tada et al., 1990; Christou et al., 1991, 1995, 1996; Datta et al., 1992, 1999, 2000, 2001; Li et al., 1993; Takimoto et al., 1994; Li and Murai, 1995; Zhang et al., 1996; Sivamani et al., 1996; Tada et al., 1996; Stark-Lorenzen et al., 1997; Chen et al., 1998; Vain et al., 1998, 2002; Kohli et

al., 1999; Ku et al., 1999; Nandadeva et al., 1999; Nishizawa et al., 1999; Tang et al., 1999; Gahakwa et al., 2000; Schaffrath et al., 2000; Konduru and Michael, 2001; Labra et al., 2001; Takahashi et al., 2001; Iwai et al., 2002; Kanzaki et al., 2002), tobacco (Deineko et al., 2000; Jach et al., 1995; Kellmann et al., 1996; Li et al., 2001; Lusso and Kuc, 1996; Melchers et al., 1993; Park et al., 1996; Rajasekaran et al., 2000; Roby et al., 1990; Tepfer et al., 1998; Yoshikawa et al., 1993), bean (Arago et al., 1996; McCabe et al., 1988; Hoffman et al., 1999; Santarem et al., 1998), maize (Fromm et al., 1986, 1990; Gordon-Kamm et al., 1990; Lyznik et al., 1993), cotton (McCabe et al., 1993; Zapata, 1999), canola (Wang et al., 1999), potato (Ray et al., 1998; Gao et al., 2000), sweet potato (Prakash and Varadarajan, 1992), wheat (Bieri et al., 2000; Clausen et al., 2000; Leckband and Lörz 1998; Oldach et al., 2001; Takumi and Shimada 1996; Vasil et al., 1993), barley (Leckband and Lörz 1998; Nuutila et al., 1999), cucumber (Punja and Raharjo, 1996; Tabei et al., 1998), and spruce (Bommineni et al., 1993), to obtain desirable characteristics such as herbicide, disease, and insect resistance in order to reduce the use of pesticides and increase yields.

According to Byrne et al. (2001), many countries in the world plant transgenic crops such as soybean, corn, cotton, canola, and potato, among which the United State had the most acreage planted to transgenic crops. Argentina was second followed by Canada and China in planting transgenic crops (Table 1.1). The most important transgenic crop in terms of acreage planted in the world was soybean, followed by cotton, canola and corn (Figure 1.1). The acreage planted to transgenic soybean and cotton increased greatly from 1999 to 2001, with the acreage planted to transgenic soybean increasing the most during those 3 years (Byrne et al., 2001). Worldwide production figures for transgenic crops, including soybean, corn, cotton and canola are shown in Table 1.2. The transgene character planted the most was herbicide resistance followed by insect

resistance. Combinations of herbicide and insect resistance occupied the third highest acreage. The acreage in virus resistant transgenic crops was also significant (Table 1.2).

The adoption of transgenic crops in the United States has been far greater than in most other countries (Figure 1.2). The percent of acreage increased more than 50% for both cotton and soybean and more than 10% for corn from 1996 to 2001 (Byrne et al., 2001). This indicates that transformation technology is being used commercially and has had a great impact on agriculture in the world. Acreage of transgenic crops may surpass non-transgenic crops in the near future providing the food and fiber necessary for human beings and livestock.

Three major methods have been used for transferring alien genes to plant tissue. Microprojectile bombardment through the use of Biolistic® devices has been used widely for transferring alien DNA to plant cells (Kikkert, 1993, Klein, et al., 1987; Hagio et al., 1991; Bommineni et al., 1993; McCabe and Martinell, 1993; Aragao et al., 1996; Kohli et al., 1999; Snyder et al., 1999). *Agrobacterium tumefaciens* has also been used widely for plant transformation (Shimamoto et al., 1989; Santarem et al., 1998; Snyder et al., 1999; Zapata, 1999; Zuker et al., 1999; Datta et al., 2000; and Labra et al., 2001). Electroporation has been used in a limited way for transforming crop plants (Fromm et al., 1986; Toriyama, et al., 1988; Tada et al., 1990; Wu et al., 1999; He et al., 2001).

Plant tissues that have been used for transformation are protoplasts (Toriyama et al., 1988, Lyznik et al., 1989; Shimamoto et al., 1989, Joersbo et al., 1990), scutellar tissues (Takumi and Shimada, 1996), meristems (McCabe et al., 1988, 1993), suspension cells (Hebert et al., 1993; Zhang et al., 1996; Nandadeva et al., 1999), immature embryos (Vasil et al., 1993; Christou et al., 1991, 1995a), mature seeds (Christou et al., 1995b), immature cotyledons (Santarem et al., 1998), and young inflorescences (Aldemita et al., 2001).

Table 1.1. Area planted in transgenic crops by country (from Byrne et al., 2001).

Country	Area planted in 2000 (millions of acres)	Crops grown
USA	74.8	soybean, corn, cotton, canola
Argentina	24.7	soybean, corn, cotton
Canada	7.4	soybean, corn, canola
China	1.2	Cotton
South Africa	0.5	corn, cotton
Australia	0.4	Cotton
Mexico	minor	Cotton
Bulgaria	minor	Corn
Romania	minor	soybean, potato
Spain	minor	Corn
Germany	minor	Corn
France	minor	Corn
Uruguay	minor	Soybean

Table 1.2. Area planted worldwide to transgenic crops and transgene traits (from Byrne et al., 2001).

Crop	Area planted in 1999 (millions of acres)
Soybean	53.4
Corn	27.4
Cotton	9.1
Canola	8.4
Potato	0.3
Squash	0.3
Papaya	0.3
Trait	
Herbicide tolerance	69.4
Bt insect resistance	22.0
Bt + herbicide tolerance	7.2
Virus resistance	0.3

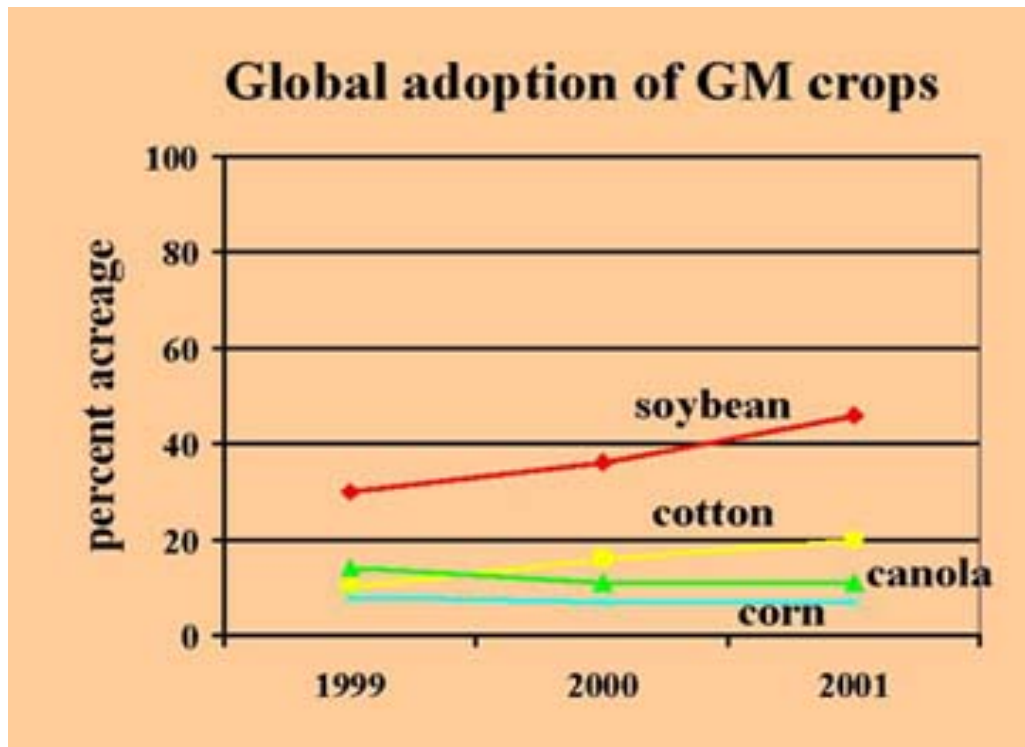


Figure 1.1. Percent of crop acreage in the world planted to transgenic crops from 1999 to 2001 (from Byrne et al., 2001).

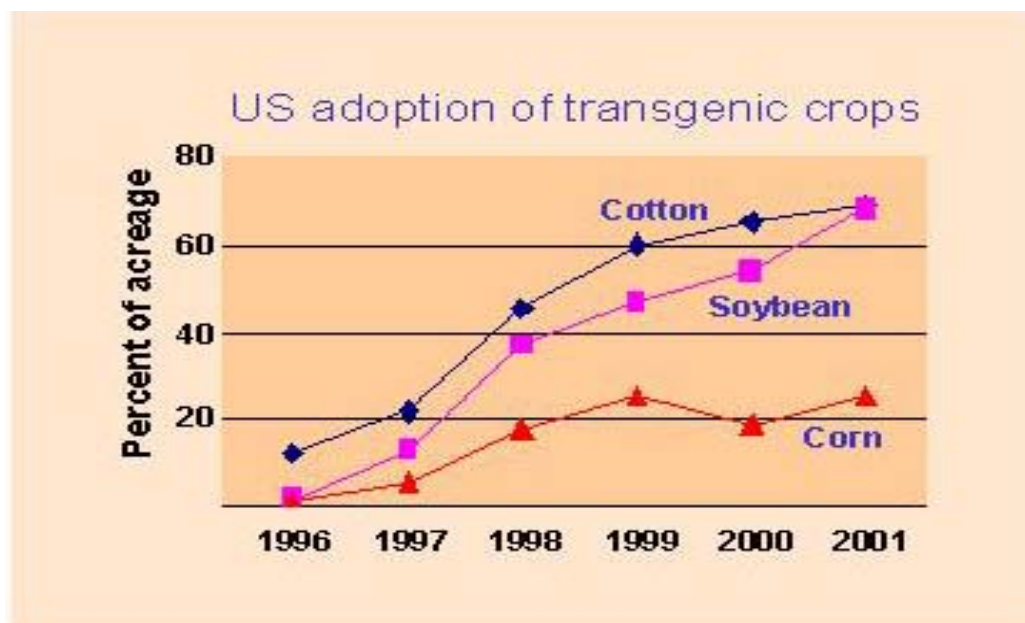


Figure 1.2. Percentage of the acreage of three major crops planted in transgenic crops in the United States from 1996 to 2001 (from Byrne et al., 2001).

Genetic transformation of rice has been an important area of research with genes for insect resistance, fungal disease resistance, virus resistance, herbicide resistance, bacterial disease resistance and nematode resistance (Ignacimuthu et al., 2000; Datta et al., 1999; 2001; Krishnamurthy and Michael, 2001; Iwai et al., 2002).

1.4 TRANSFORMATION USING PATHOGENESES-RELATED (PR) PROTEIN GENES

The incorporation of disease resistance when developing improved crop cultivars is one of the major challenges for plant breeders, as diseases cause major yield losses and have impacted humans worldwide (Agrios, 1997). Using conventional breeding methods, such as crossing and selection, to incorporate desired disease resistance genes into agronomically and horticulturally important crops has been highly successful and provides a major component to IPM in most crop plants. Increasing resistance to SB of rice has been achieved to a limited extent using sources of partial resistance (Rush et al., 1996, 2002; Sha, 1998). With the beginning of the molecular era of plant biology in the early 1980's, identifying, cloning and characterizing plant disease resistance genes has become a major research area (Punja 2001, Crute and Pink, 1996). Over the past 10 years, many mechanisms of plant response to pathogen infection have been identified (Nicholson and Hammerschmidt, 1992; Crute and Pink, 1996; Donofrio and Delaney, 2001). After the identification of these genes, their specific roles and importance in disease response pathways were evaluated using transgenic plants and genetic engineering techniques (Neuhaus et al., 1991; Beffa et al., 1996; Bieri and Fütterer, 2000; Powell et al., 2000).

Punja (2001) summarized cloned genes into five general categories according to the plant's responses to pathogen infection:

- 1) The expression of gene products that are directly toxic to pathogens or reduce their growth, which includes pathogenesis-related proteins (PR proteins), such as hydrolytic enzymes

(chitinases, glucanases), antifungal proteins (osmotin-, thaumatin-like), antimicrobial peptides (thionins, defensins, lectin), ribosome inactivating proteins, and phytoalexins.

2) The expression of gene products that destroy or neutralize a component of the pathogen defense arsenal such as polygalacturonase, oxalic acid, and lipase. For example, the expression of oxalate oxidase protein in barley is a response to attack by the pathogen *Esrysiphe graminis* f. sp. *hordei*.

3) The expression of gene products that can potentially enhance the structural defenses in the plant. These include elevated levels of peroxidase and lignin.

4) The expression of gene products releasing signals that can regulate plant defenses. This includes the production of specific elicitors, hydrogen peroxide, salicylic acid, and ethylene.

5) The expression of resistance gene products involved in the hypersensitive response and in interactions with avirulence factors.

In recent years, the expression of PR proteins in transgenic plants has become a useful technology to obtain resistance. Chitins and glucans are major components of the cell wall of most higher fungi, and chitinase and β -1,3-glucanase are capable of degrading fungal cells and exhibit antifungal activity *in vitro* (Punja and Zhang, 1993; Boller, 1993; Lusso and Kuć, 1996;).

1.4.1 Plant Chitinases and β -1, 3-glucanases

Chitinases and β -1, 3-glucanases have the ability to catalyze the hydrolysis of chitin and β -1, 3-glucan, major components of the cell wall of most filamentous fungi except for the Oomycetes (Broglie et al., 1991). The products formed are oligosaccharides and it is possible that such oligosaccharides are perceived by the plant cell as signals or elicitors to induce active defense responses. For example, soybean cells react to small glucan elicitors derived from cell walls of the pathogen *Phytophthora megasperma* (Boller, 1993). The expression of the class II

chitinase gene, *A.h.Chi2;1*, from peanut in transgenic tobacco plants was triggered by substances very quickly excreted from germinating conidia or by growing hyphae. The expression of two class II chitinase genes from strawberry plants was induced by *Colletorichum fragariae* or *C. acutatum* (Khan and Shih, 2004).

1.4.2 Plant Chitinases

Based on the deduced amino acid sequence, plant chitinases have been classified into seven groups (Neuhaus, 1999). Class I chitinases have N-terminal cysteine-rich domains homologous to hevein, with many of them having high isoelectric points above pH 9.0. Class II chitinases have homology to class I chitinases, but no cysteine-rich domain at the N-terminal end and a short extension at the C-terminal end. Many of them have low isoelectric points below pH 5.0. Class III chitinases have isoelectric points above pH 9.0 and below pH 5.0.

Class I chitinase from tobacco in transgenic *Nicotiana sylvestris* was shown to be the limiting factor in the defense reaction against the pathogen *Cercospora nicotianae* (Neuhaus et al., 1991).

Two class II chitinases from strawberry plants were induced by two important fungal pathogens *C. fragariae* and *C. acutatum*, and the relative quantity of the mRNA was different in response to the two fungi as detected by real time reverse transcription PCR (Khan and Shih, 2004).

Plant chitinases are potent inhibitors of fungal growth induced in response to the plant hormone ethylene or by infection by fungal pathogens (Schlumbaum, et al, 1986). They have the antifungal function to hydrolyze the chitin polymer to release N-acetyl glucosamine oligomers, and to cleave the β -1, 4 bonds in fungal cell walls.

Chitinases have been intensively studied (Punja and Zhang, 1993; Kellmana et al., 1996). These enzymes may be expressed constitutively at low levels, but are dramatically enhanced by abiotic and biotic factors (Punja and Zhang, 1993; 1996; Roby et al., 1990).

Oilseed rape transformed with a tomato chitinase gene showed increased resistance to the fungal pathogens *Cylindrosporium concentricum*, *Phoma lingam* and *Sclerotinia sclerotiorum* (Grison et al., 1996). Transgenic cucumber plants transformed with the class I rice chitinase gene (*RCC2*) showed enhanced resistance to gray mold caused by *Botrytis cinerea*. The disease resistance was confirmed to be heritable, so the highly resistant transgenic cucumber strains should serve as good sources for disease resistance (Tabei et al., 1998). Yamamoto et al. (2000) also showed that transgenic grapevine plants, expressing the same chitinase gene, enhanced resistance to powdery mildew caused by the fungal pathogen *Uncinula necator*. Nishizawa et al. (1999) transferred the class I rice chitinase gene into Japonica rice varieties. The transgenic rice plants which expressed the rice chitinase gene showed significantly higher resistance against the rice blast pathogen *Magnaporthe grisea*, and the high-level expression of the transgene and blast resistance were stably inherited by the next generation. The rice class I chitinase gene was also transferred to rice using *Agrobacterium tumefaciens*. Bioassay showed that transgenic plants restricted the growth of *R. solani* (Datta, et al., 2000). Datta et al. (2001) transferred another class I chitinase gene (*RC7*) to indica rice cultivars. The transformants synthesized different levels of chitinase proteins compared to the normal rice chitinases, and showed different levels of enhanced resistance when inoculated with *R. solani*.

In bean leaves, class I chitinase has been found to localize in the vacuolar compartment, and the enzyme is a 30-kD protein that catalyzes the hydrolysis of chitin (Broglie et al., 1989; Roby et al., 1990; Boller, 1993). The gene was sequenced by Broglie et al., (1989) and

expression of this gene was shown to be dependent upon either exogenous ethylene (Schlumbaum, et al., 1986) or oligosaccharide elicitors.

The expression of bean chitinase in transgenic tobacco plants gave resistance to *R. solani*, and the inhibition of pathogen growth by this chitinase *in vitro* was due to the disruption of growing fungal-hyphal tips (Broglie et al., 1991; Boller, 1993). The transgenic tobacco plants showed a high-level localized induction of chitinase promoter activity in response to infection by the phytopathogens *B. cinerea*, *S. rolfii*, and *R. solani* (Roby et al., 1990), which suggested that infection triggered the expression of resistance.

1.4.3 Plant β -1, 3-glucanases

Based on the deduced amino acid sequences of β -1, 3-glucanases from tobacco, β -1, 3-glucanases have also been grouped into three classes (Boller, 1993). Although there are fewer examples of the expression of glucanases in transgenic plants (Punja, 2001), the expression of glucanases in transgenic plants was demonstrated to reduce disease symptoms caused by fungal pathogens in a manner similar to that for chitinase expression (Mauch et al. 1989, Lusso et al. 1996, Yoshikawa et al. 1993).

Transgenic tobacco plants expressing soybean β -1, 3-endoglucanase showed a high level of disease resistance against *Phytophthora parasitica* var. *nicotianae* and *Alternaria alternata* tobacco pathotype. The enzyme did not directly inhibit several fungal pathogens *in vitro*, but generated elicitor signals leading to active disease resistance (Yoshikawa et al., 1993). Yoshikawa et al. (1993) also indicated that transgenic tobacco plants with soybean β -1, 3-endoglucanase transgene showed a high correlation between the enzyme activity and resistance to the fungal pathogen. This supported the hypothesis that the resistance against *Peronospora tabacina* and *P. parasitica* var. *nicotiana* was due to the activity of

β -1, 3-glucanase (Lusso and Kuć, 1996).

Bioassays with fungi growing in artificial media have clearly demonstrated the antifungal potential of plant chitinases and β -1, 3-glucanases (Boller, 1993). Fungi are temporarily inhibited, but with the capacity to adapt to high levels of these two enzymes. Therefore, to express antifungal activity, it might be important to increase the concentrations of these enzymes rapidly in the vicinity of an approaching hypha. This is likely to happen naturally in the hypersensitive response (Boller, 1993).

Tobacco β -1, 3-glucanase is in class I and belongs to the pathogenesis-related protein Family, PR-2, which is located in the cell vacuole (Melchers et al., 1993; Loon and Strien, 1999). This enzyme showed antifungal activity against *Fusarium solani* where it lysed hyphal tips and inhibit growth (Sela-Buurlage et al., 1993), but different class of chitinases and β -1, 3-glucanases gave different levels of resistance to specific fungi. Sela-Buurlage et al. (1993) indicated that only the class I-type tobacco chitinase and β -1, 3-glucanase exhibited antifungal activity against *F. solani in vitro*, but the class II β -1, 3-glucanases had no activity against this pathogen.

1.4.4 Combinations of Chitinase and β -1, 3-glucanase

The expression of two or more antifungal genes in transgenic crops may provide more effective disease control than a single-gene. *In vitro* antifungal assays of tobacco class I chitinase and β -1, 3-glucanase, used singly or combined, showed that the two enzymes acted synergistically (Sela-Buurlage et al., 1993). The combined expression of class I chitinase and class I β -1, 3-glucanase genes in transgenic tomato gave increased resistance to *F. oxysporum* f.sp. *lycopersici* (Jongedijk et al., 1995). The expression of rice class I chitinase gene and the alfalfa class II glucanase gene by constitutive co-expression in transgenic tobacco resulted in

substantially greater protection against the fungal pathogen *Cercospora nicotianae*, causal agent of frogeye leafspot, than either transgene alone (Zhu et al., 1994).

Not only the expression of class I enzymes gave resistance to fungal diseases, but also class II enzymes. Class II chitinase and class II β -1, 3-glucanase from barley were transferred to tobacco by transformation. The expression of the individual genes showed an increase in resistance to *R. solani*, while the co-expression of the two genes produced significantly enhanced protection against fungal attack (Jach et al., 1995). This again indicated that a synergistic protective interaction of the co-expressed anti-fungal proteins occurred *in vivo*.

Although Class I tobacco chitinase and β -1, 3-glucanase acted synergistically, the class II chitinase showed limited antifungal activity when combined with higher amounts of class I β -1, 3-glucanase *in vitro* (Sela-Burrlage et al., 1993).

Co-transformation of rice with a class I bean chitinase and class I tobacco β -1, 3-glucanase for rice sheath blight control has not been reported and is the major objective of our research.

1.5 PLANT THIONINS

Plant thionins are small, basic, cysteine-rich antimicrobial proteins that are toxic in various biological systems where they destroy pathogen membranes. This process plays a role in plant defense (Epple et al., 1997; Bohlmann, 1994). The over-expression of Arabidopsis thionin *Thi2.1* gene in transgenic Arabidopsis enhanced resistance against *F. oxysporum* f. sp. *matthiolae* (Epple et al., 1997).

It has been demonstrated that the endogenous rice thionins, *Osthi1*, do not have enough activity to protect against bacterial infection. However, the overproduction of the oat thionin

Ashthi1 in Japonica rice plants resulted in strong resistance to infection by *Burkholderia plantarii* and *B. glumae* (Iwai et al., 2002).

Purothionins and hordothionins extracted from wheat and barley flour, respectively, were toxic to the bacterial pathogens *Clavibacter michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus*, and *Xanthomonas campestris* pv. *vesicatoria* with minor differences for different strains (Florack et al., 1993). The expression of hordothionin from barley endosperm in transgenic tobacco enhanced resistance to *Pseudomonas syringae* pv. *tobaci* 153 and *P. syringae* pv. *syringae* (Florack et al., 1994).

Leaf thionins of barley can be induced by infection with powdery mildew and they are incorporated into papillae produced as a defense against the pathogen (Bohlmann, 1994). Jasmonic acid and its methyl ester are also involved in the same stress related reaction as the pathogen-induced leaf thionins of barley (Andressen et al., 1992). This mechanism of thionins in defense against plant pathogens theoretically could be used to enhance the resistance of transgenic plants of other crops.

Transformation with the barley leaf thionin gene to rice to obtain resistance to bacterial panicle blight (seedling and grain rot) has not been reported and is an objective of our research.

1.6 GENE SILENCING

Transgenic plants having the transgene, but without expression of the desired resistance were probably affected by gene silencing. Gene silencing is a common phenomenon in transgenic plants. Gene silencing includes transcriptional gene silencing and post-transcriptional gene silencing. Transcriptional gene silencing is due to transcription inactivation by promoter methylation. The methylation of the transgene sequence could be decreased in the transgenic plants treated with 5-azacytidine (Kohli et al., 1999; Wang and Waterhouse, 2000). Post

transcriptional gene silencing is due to the specific degradation of RNA (Smith et al., 2000). The transgene copy number and the inserted position are often the reason for post transcriptional gene silencing (Baulcombe, 1996; Buch et al., 2001; Wang and Waterhouse, 2000).

1.7 THE POLITICAL AND SCIENTIFIC ISSUES OF USING GENE TRANSFORMATION AND GENETICALLY MODIFIED PLANTS

The major differences between conventional breeding and genetic engineering of crop plants were listed by Rene Custers (2001) as:

“1. With genetic engineering it has become possible to transfer a single gene (or a specific number of genes) into a crop, while in conventional breeding large parts of the plant genome are changed. Genetic engineering enables breeders to selectively introduce the characteristics which are of interest and to avoid the introduction of undesired characteristics”.

“2. Conventional breeding is limited to breeding within plant families. Genetic engineering is not limited to species barriers. Genes found in bacteria, or in any other organism, can be engineered into a crop plant”.

There are considerable concerns about the impact of genetically modified (GM) crops throughout the world (Punja, 2001). Key issues in the environmental assessment of GM crops are invasiveness, vertical or horizontal gene flow, other ecological impacts, effects on biodiversity, the impact of the presence of GM gene products in products from non-transgenic plants, toxicity and food safety of genetically engineered crops, and allergenicity of foods derived from genetically engineered crops (Conner et al., 2003; Rene Custers 2001). Studies of the transgenic canola crop (oilseed rape) showed that gene flow from these plants to non-transformed rape plants takes place through outcrossing pollen during sexual reproduction. There is also potential for spread of transgenes to closely related weedy species impacting, for instance, weed control

with herbicides (Daniell, 1999). This means that risk assessments have to be considered when releasing crop varieties with transgenes (Custers, 2001; Orson, 2002).

Generally, the impacts of GM crops are very similar to the impacts of new cultivars derived from traditional breeding (Conner, 1997). But “whenever questions arise concerning use of GM crops, science-based evaluations should be used on a case-by-case approach (Conner et al., 2003)”. Also, taking into account factors such as the genes inserted, the nature of the target crop, local agricultural practices, agro-ecological conditions, and trade policies is very important (Conner et al., 2003; Nuffield Council on Bioethics, 2003).

The Royal Society (1998) report on GM crops concluded that “there was no evidence for transfer of intact genes to humans either from bacteria in the gut or from foodstuffs, except for the consumption of DNA, which has shown no significant risk to human health. Ingestion of GM crop DNA has not been shown to have any deleterious effects”.

1.8 OBJECTIVES OF THIS STUDY

The objectives of this research were to: 1) use co-transformation to co-transfer the plasmid pChiHy with a bean chitinase gene (Broglie et al., 1989), the *hpt* gene in the plasmid pGluHy (obtained from Dr. D. Shih’s laboratory in the Department of Biology of Louisiana State University), along with a tobacco β -1, 3-glucanase gene and *hpt* gene, and the plasmid pUBIBarHy (Obtained from Dr. Shih’s laboratory), with the *bar* gene for resistance to Liberty herbicide (common name: glufosinate-ammonium) and the *hpt* gene into rice callus, regenerate rice plants, and test the plants for resistance to sheath blight, Liberty herbicide resistance, and hygromycin B resistance, 2) to co-transform the plasmid pMTHy (obtained from Dr. Shih’s laboratory) containing the barley thionin gene and the *hpt* gene and the plasmid pUBIBarHy with the *bar* and *hpt* genes into rice callus, regenerate rice plants, and test the plants for resistance to

bacterial panicle blight, Liberty herbicide, and hygromycin B resistance, 3) develop molecular evidence to prove that the genes were transferred to the plants showing the various resistances. PCR and southern blot analysis will be used to carry out this objective.

CHAPTER 2

CO-TRANSFORMATION OF CHITINASE, β -1, 3-GLUCANASE AND *bar* GENES TO TAIPEI 309 FOR IMPROVING SHEATH BLIGHT RESISTANCE IN RICE

2.1 INTRODUCTION

Transfer of multiple genes by repetitive insertion of single coding sequences is impractical due to the time and effort required for recovery of transgenic tissues with multiple transgenes. Chen et al. (1998) reported that after co-transformation using a mixture of genes in 14 different plasmids, 85% of the R₀ plants contained more than two transgenes and 17% of the plants had more than nine of the transgenes. The integration of multiple transgenes occurred at either one or two genetic loci, and in most instances inheritance conformed to a 3:1 Mendelian ratio (Chen et al., 1998; Tang et al., 1999). The plasmid with the highest molar ratio had a better chance to be inserted (Chen et al., 1998). Gelvin (1998) explained that the reasons the transgenes integrated into the same site in the plant genome were: 1) the vector sequences were the same for all the gene constructs and this could provide regions of homology for recombination either before or after DNA integration, and 2) the integration of any one gene damages plant genome DNA so that other plasmid molecules were “attracted” to this site.

Chitinases and β -1, 3-glucanases are present in higher plants where both enzymes were important antifungal proteins (Schlumbaum et al., 1986; Mauch and Staeheline, 1989). Chitinase catalyzes the hydrolysis of β -1, 4-linkages of the *N*-acetylglucosamine polymer of chitin to inhibit fungal pathogen development by lysing hyphal tips. This releases *N*-acetyl glucosamine oligomers that serve as elicitors to amplify defense response in cells surrounding a site of infection (Punja and Zhang, 1993; Datta et al., 2001).

Transgenic tobacco plants with a class I bean chitinase gene had an increased ability to survive in soil infected with the fungal pathogen *Rhizoctonia solani* and delayed development of

disease symptoms (Broglie et al., 1991), which indicated that the bean chitinase was an important enzyme for inhibiting the pathogen. Tobacco class I β -1, 3-glucanase is basic and confined to the intracellular vacuole (Melchers et al., 1993). Its antifungal activity was closely associated with high levels of the β -1, 3-glucanase (Sela-Buurlage et al., 1993; Lusso and Kuć, 1996). The co-expression and synergetic expression of chitinase and β -1, 3-glucanase genes in tomato plants enhanced their fungal resistance (Zhu et al., 1994; Jongedijk et al., 1995).

It is difficult to control sheath blight (SB) disease with pesticides or cultural practices as *R. Solani* has a broad host range (Lee and Rush, 1983), and chemical control using fungicides such as Quadris and Moncut are expensive (Groth and Rush, 1988; Groth et al., 1996). To date, no complete SB resistance has been identified, only moderated or partial resistance is available (Lee and Rush, 1983; Sha, 1998). The lack of natural complete resistance to sheath blight makes using conventional breeding methods for developing resistant varieties difficult. It appears that transferring alien genes to rice could add to the arsenal of resistance genes available to rice breeders and plant pathologists. .

The purpose of this study was to co-transfer chitinase and β -1, 3-glucanase genes into rice calli and to determine if rice plants regenerated from the calli will have higher levels of SB resistance than the non-transformed plants.

2.2 MATERIALS AND METHODS

2.2.1 Tissue Culture Media

All the chemicals for making each medium were ordered from Sigma (St. Louis, MO, USA 63178-9916), and each medium was poured into separate sterile plastic Petri dishes. Six different media were used in this research. A callus induction medium was used to induce calli from the scutella of germinating mature rice seeds, an osmotic medium was used for creating

high osmotic pressure on each cell in the calli, so that plasmid DNA can be easily taken up by callus cells. A selection medium containing the antibiotic hygromycin B was used to select transformed cells having the hygromycin resistance gene (*hpt*), a pre-regeneration medium was used so that the embryogenic calli mature and produce shoots, and a regeneration medium was used to regenerate plants from transformed calli. A rooting medium was usually required to allow plantlets produced on calli to develop roots to the point that they could be transferred to soil in the greenhouse.

The callus induction medium (Datta et al., 1990) contained 4.3g/L of Murashige and Skoog (Murashige and Skoog, 1962) basal salt mixture (MS salts), 30g/L of sucrose, 8g/L agar, 0.3g/L Casein hydrolysate, 1ml/L Gamborg's vitamins (B5 vitamins), 0.5g/L of L-proline, 0.5g/L of L-glutamine and 2mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) from a stock solution with 0.5mg/mL of 2,4-D (Datta et al., 2000).

The osmotic medium (Kikkert, 1993) contained the same chemicals and concentrations as callus induction medium plus 30g/L D-Mannitol, and 30g/L D-Sorbitol.

The selection medium contained the same chemicals and concentrations as callus induction medium plus 50mg/L hygromycin B (Datta et al., 1999).

The pre-regeneration medium (Datta et al., 1999) contained 4.3g/L of MS salts, 30.0/L sucrose, 8.0g/L agar, 0.3g/L Casein hydrolysate, 1ml/L of B5 vitamins, 0.5g/L of L-proline, 0.5g/L of L-glutamine, 2mg/L of 6-benzylaminopurine (BAP), 5mg/L of abscisic acid (ABA), 1mg/L of 1-naphthalene acetic acid (NAA), and 50mg/L hygromycin B.

The regeneration medium (Datta et al., 1990) contained 4.3g/L of MS salts, 30g/L of sucrose, 8g/L agar, 0.3g/L of casein hydrolysate, 1ml/L of B5 vitamins, 0.5g/L of L-proline, 0.5g/L of L-glutamine, 3.5mg/L of BAP, 0.5mg/L of NAA and 50mg/L hygromycin B.

The rooting medium contained 2.15g/L of MS salts, 10g/L sucrose, 8g/L agar, 1ml/L B₅ vitamins and 50mg/L of hygromycin B.

2.2.2 Plasmids Used in Transformation Experiments

There were four different plasmids, each with different transgenes, used in the transformation experiments. The plasmid pChiHy had a bean chitinase gene (approximate 1035bp) (Broglie et al., 1989) with the maize ubiquitin promoter (Christensen et al., 1992) and the *hpt* gene (Zalacain et al, 1986) with the maize ubiquitin promoter. The plasmid pGluHy had tobacco β -1, 3-glucanase gene (approximate 1093bp) with the maize ubiquitin promoter and the *hpt* gene with the ubiquitin promoter. The plasmid pMTHy had the barley leaf thionin gene (approximate 1000bp) with the 35s promoter and the *hpt* gene with the 35s promoter. The plasmid pUBIBarHy had the *bar* gene (approximate 615bp) from *Streptomyces hygroscopicus* with the maize ubiquitin promoter and the *hpt* gene with the ubiquitin promoter (Wohlleben et al, 1988).

Each plasmid was transformed to competent cells of *Escherichia coli*. All the plasmids were provided by Dr. Ding S. Shih's laboratory in the Biochemistry section of the Biological Sciences Department at Louisiana State University in Baton Rouge, Louisiana.

2.2.3 Callus Induction

Dehulled rice seeds were sterilized for 30 minutes in a solution with 1.5% sodium hypochlorite in a beaker with a magnetic stirring rod. The rice seeds were then washed three times with sterile water under a laminar flow hood. The sterilized rice seeds were inserted into callus induction medium with the embryo side up using sterile technique (Figure 2.1) and the dish with rice seeds were incubated in the dark at 27C. After 3 weeks culture in the dark, calli were induced from the scutella of the rice seeds. Pieces of calli were then transferred to fresh

callus induction (CI) medium, incubated in the dark at 27C and subcultured every 2 weeks on CI medium. The callus type induced on CI medium is shown in Figure 2.2 under a stereoscopic microscope. After 2 to 3 months of subculturing, rapidly growing rice calli were separated into small pieces and placed onto osmotic medium for 24 hours in the dark at 27C. These calli were then transferred to CI medium for immediate transformation with 90 to 100 pieces of callus in each dish (Figure 2.3).

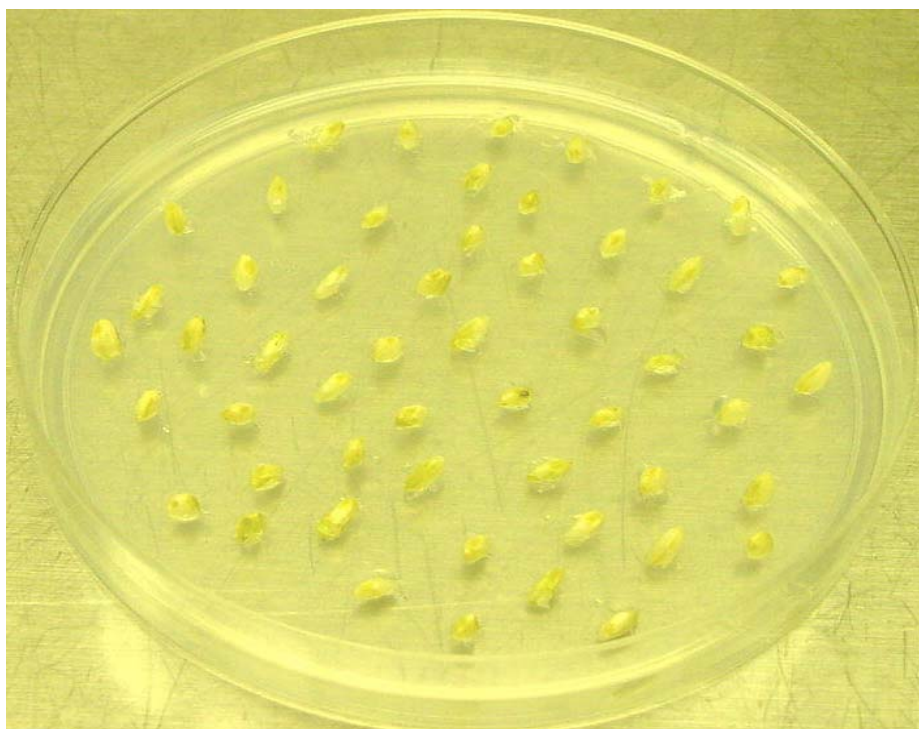


Figure 2.1. Surface sterilized, dehulled seeds plated onto callus induction medium.



Figure 2.2. Callus produced on callus induction medium and ready to transfer to the osmotic medium.

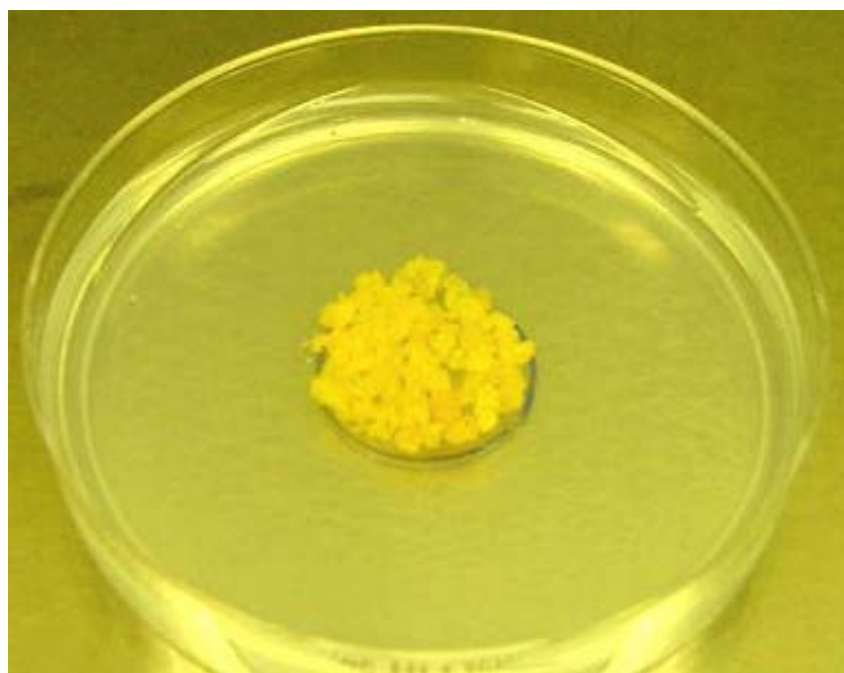


Figure 2.3. Calli transferred from the osmotic medium to callus induction medium for transformation. Calli were closely packed in a 25 mm diameter mass in the center of the plate to provide a suitable target for the DNA coated gold particles fired from the biolistic® PDS-1000 / He device.

2.2.4 Procedure for Transformation of Rice Calli Using Plasmid DNA

A Biolistic® PDS-1000/He device from BioRad™ was used for transferring the plasmids to rice calli (Kikkert, 1993). Fin-pipettes, filter paper, stopping iron screen, and iron baffle screen were autoclaved before each transformation. Retaining cap and rupture disks were placed in 70% ethanol for 30 minutes and air dried. The accelerator tube and the chamber of the gene gun were sprayed with 70% ethanol.

The main screw and gauge of the helium supply tank were opened to adjust the outlet pressure at 1700psi. A vacuum pump was used so that the vacuum level in the chamber was 26 to 28 mm Hg. A rupture disk was placed on a retaining cap, and the retaining cap was screwed to the end of the gas acceleration tube. A stopping screen was placed into the microcarrier launch assembly. The entire assembly was placed into the chamber, and VAC switch was pressed to get vacuum level 26-28 mm Hg. The VAC switch was put in the hold the position and the FIRE button was pressed and held. These procedures were described in the manual for the biolistic® PDS-1000 / He device (Kikkert, 1993).

The 25mm diameter macrocarriers and rupture disks, which would rupture at 1550psi pressure, were sterilized with 70% ethanol and dried inside the laminar flow hood.

Ten mg of gold particles (1.5-3.0uM from BioRad™) were sterilized in 70% ethanol in a micro-centrifuge tube for 15 minutes while vortexing on a Vortex-Genie™ at it's maximum speed. The microcentrifuge tube was centrifuged for 5 minutes at 14,000rpm with an Eppendorf microcentrifuge, and the ethanol was discarded. The sterilized gold particles were washed two times with sterile water. The following materials were added to the tube with sterile gold particles to form the transforming mixture: 180uL 50% glycerol, three plasmids pUC8, pCluHy and pChiHy containing the *bar*, β -1, 3-glucanase and chitinase genes respectively, in the same

molar ratio, 180uL CaCl_2 , and 180uL 0.1M spermidine. The mixture was vortexed for 10 minutes so that the gold particles were coated with the mixture of plasmid DNAs. The mixture was centrifuged with a microcentrifuge for 5 seconds at 14,000rpm, and the supernatant was removed. The DNA-coated gold particles were washed once with 500uL 70% ethanol and once with 500uL 100% ethanol. Then 120uL 100% ethanol was added to the DNA-coated gold particles and 6uL of the re-suspended gold particles were dispensed onto a Kapton macrocarrier disk (25mm in diameter from BioRadTM).

Using the procedure described for the Biolistic® PDS-1000 / He device (Kikkert, 1993), the plasmids were transferred at high speed to the osmotic treated calli. Each dish of calli was bombarded once, the treated (transformed) calli were left on the same dish overnight to recover, and then the transformed calli were transferred to selection medium the next day.

2.2.5 Selection of Transformed Calli and Regeneration of Transformed Plants

Calli were transferred to fresh selection medium every 10-14 days depending on the condition of calli (Figure 2.4). After subculturing four times on selection medium, transformed calli were transferred to pre-regeneration (PR) medium and cultured in the tissue culture room using a 12h light and 12h dark regime at 27°C. After the calli were incubated in PR medium for 10-14 days, they were transferred to regeneration medium and maintained in the same tissue culture room. These calli were subcultured on regeneration medium every 2 weeks. Green spots appeared on the cultured calli on regeneration medium in about 20 days (Figure 2.5), then some of the green spots developed into plantlets (Figure 2.6). After the shoots had developed to the 2-leaf stage they were transferred to rooting medium (Figure 2.7). After a root system that would support transplanted plants was established, the plants were transferred to 20cm diameter black plastic pots with a potting soil mixture made up of soil: sand: peat moss (1:2:1) in the

greenhouse. As the plants were transferred to the greenhouse at different times, 101 transgenic Taipei 309 plants were transplanted to the field at the LSU Rice Research Station at Crowley, LA on May 21, 2003, and the rest of plants were kept in the greenhouse.

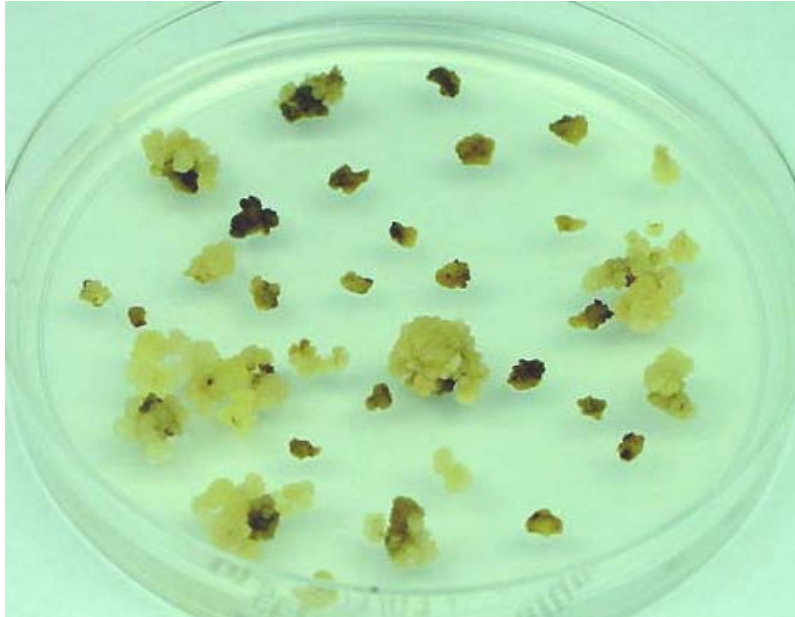


Figure 2.4. Calli on selection medium containing 50ppm hygromycin B. Brown areas on calli were cells killed by exposure to the hygromycin.

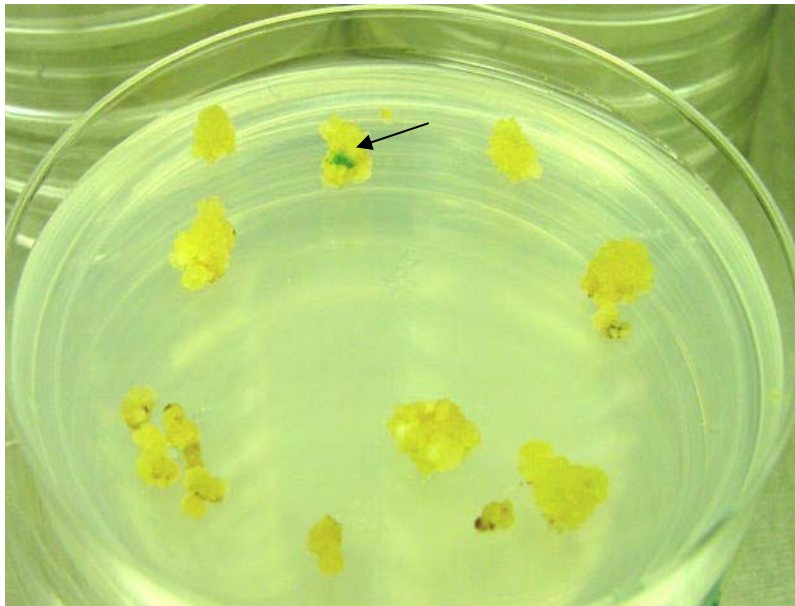


Figure 2.5. Calli on regeneration medium with 50ppm hygromycin B. Green spots were precursors to shoot formation.

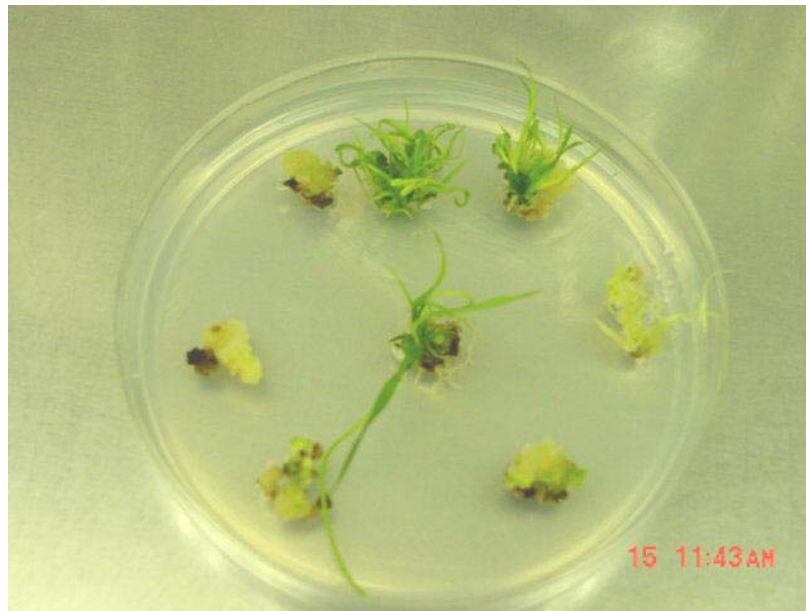


Figure 2.6. Calli forming shoots on regeneration medium with 50ppm Hygromycin B.



Figure 2.7. Plantlets were transferred to rooting medium.



Figure 2.8. After plantlets established root systems, they were transferred to the greenhouse.

2.2.6 Testing Transformed Plants for Hygromycin B Resistance

All of the plasmids used in these transformation studies had the *hpt* gene for resistance to hygromycin B for use as a selectable marker (Ortiz et al., 1996). All transformed plants were regenerated on media containing hygromycin B and were tested to see if they had an expressing *hpt* gene. The transgenic plants C4-3 and C9-1, provided by Dr. Q.M. Shao and with known resistance to hygromycin B (expressing the *bar* gene), were used as the positive control, non-transgenic plants from seeds of the variety Taipei 309 were used as the negative control, and transgenic plants from our greenhouse and field tests were tested for resistance to hygromycin B.

The concentration of hygromycin B used in the tests was 200ppm ai in sterile water. The tips of leaves on plants to be tested were dipped into the hygromycin B solution and the leaf was cut 10-20mm from the tip in a straight line across the blade, with scissors, while immersed. The remaining portion of the leaf was held in the solution for 5 seconds. Data on resistance was collected 5 days after this treatment. The distance in mm from the cut area (blue arrow) to the

end of the necrotic lesion that formed across the leaf blade (purple arrow) was recorded as the lesion length, and from the cut area to the end of any necrosis produced by the antibiotic (red arrow) was recorded as the extended lesion length (Figure 2.9).



Figure 2.9. Primary necrotic lesion and extended lesion produced by hygromycin B on non-transgenic plants or transgenic plants not expressing the *hpt* gene. Note the reaction of the hygromycin resistant control plant on the left.

2.2.7 Testing Transformed Plants for Resistance to Liberty Herbicide

One of the plasmids used in these co-transformation experiments had the *bar* gene for resistance to Liberty herbicide. As in the hygromycin B resistance screening test, transgenic plants from Dr. Q.M. Shao's transgenic lines C4-3 and C9-1 were used as the positive Liberty herbicide resistance control, non-transgenic plants of Taipei 309 served as the non-Liberty resistance control, and transgenic plants were tested in greenhouse and field tests for resistance to Liberty herbicide.

The Liberty solutions used in the tests had 363ppm ai Liberty herbicide with 1g/L AlconoxTM detergent powder in sterile water. Some transgenic plants, positive control plants and

non-transgenic Taipei 309 plants were chosen to test for Liberty resistance using 750ppm ai Liberty. The same leaf tip immersion/cutting technique used to test transgenic plants for resistance to hygromycin B was used to test for Liberty resistance. Cut leaf tips were held in the Liberty solution for 5 seconds. The resistance level was determined 5 days after treatment of putative transgenic plants. The distance from the cut end of the leaf blade (blue arrow) to the end of the primary lesion (purple arrow) was designated as the length of the necrotic lesion and the length of the lesion in mm from the cut end of the leaf to the maximum lesion extension (red arrow) was the extended lesion length (Figure 2.10).



Figure 2.10. Primary lesion length and extended lesion length after treatment with Liberty herbicide.

2.2.8 Testing Transgenic Plants for Increased Sheath Blight Resistance

Transgenic plants were co-transformed with the PR genes for chitinase and beta glucanase production. It was necessary to test these plants for changes in resistance to the rice SB disease. Theoretically, each transgenic plant could have both of these genes, one of the genes,

or none of the PR genes for resistance to fungal diseases. Also, the insertion site, the number of times each gene was inserted, and the expression status of each gene in the plant could affect the expression of SB resistance. The production of clonal variation for increased resistance or susceptibility was also a possibility. These factors made it very important to obtain an accurate evaluation of the sheath blight resistance level in each plant putatively transgenic with the two PR genes. The inoculation method used was reported by Sha (1998). The pointed ends of round wooden toothpicks were cut 0.5cm from each end and washed with tap water. A 4-ml volume of toothpicks tips was mixed with 10ml of potato dextrose broth (PDB) medium (Difco) in glass Petri dishes. The Petri dishes were autoclaved at 121°C for 30 minutes, allowed to cool and inoculated with plugs of *R. solani* (isolate LR-172) in a laminar flow hood (Figure 2.11). The toothpick pieces absorbed the PDB medium during autoclaving. Inoculated toothpick tips were cultured at room temperature for 7-10 days, so that the fungus could grow into the toothpick tips. One toothpick tip served as inoculum to inoculate a single tiller on a transgenic plant. The inoculum was inserted behind a fully extended leaf sheath just behind the ligule at the collar (Figure 2.12).

In both field and greenhouse tests, non-transgenic Taipei 309 plants were used as susceptible controls and non-transgenic plants of the sheath blight susceptible variety Cocodrie were used to determine if the environmental conditions during the test were optimum for SB development. The heights of inoculated transgenic and non-transgenic tillers were measured in cm, from the soil surface to the flag leaf collar, and the height of SB lesion development on each inoculated plant was also measured. The ratio of plant height to lesion height was calculated as lesion height/plant height.



Figure 2.11. *Rhizoctonia solani* inoculum was prepared on 0.5cm toothpick tips.

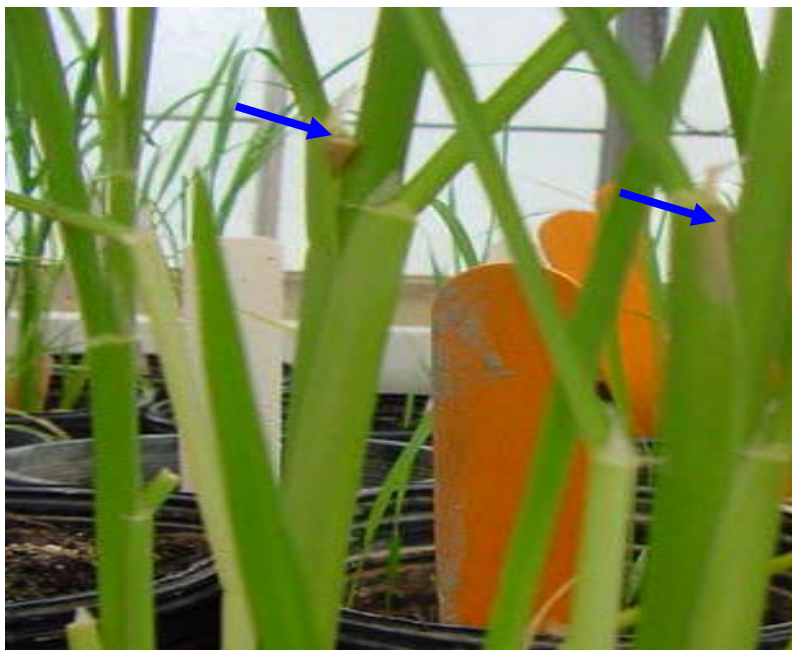


Figure 2.12. The toothpick inoculum was inserted behind a fully extended leaf sheath just behind the ligule at the collar.

2.2.8.1 Greenhouse Test

Plants remaining in pots in the greenhouse were inoculated at the maximum tillering stage 50-60 days after being transferred to the greenhouse. Plants were inoculated (6/10/03) using the toothpick inoculation method in the collar of the last fully expanded leaf. After inoculation, a plastic cover was placed over the bench to form a humidity chamber (Figure 2.13). For the development of SB, plants were kept inside the chamber after inoculation. The plastic cover was pulled down in the evening to form a closed chamber, and one side of the plastic was pulled up 30cm in the morning so that the temperature in the chamber remained moderate. Three weeks after inoculation (6/30/03), the SB lesion length and plant height were measured.



Figure 2.13. Plastic cover in greenhouse to form humidity chamber over inoculated plants.

2.2.8.2 Field Test

Transgenic and non-transgenic plants were transplanted from the greenhouse to the field at the Rice Research Station, Crowley, LA in May 21, 2003, and plants were fertilized with NPK (24-13-13). The row spacing was 25.4cm and plant spacing was 10cm. One month after transplanting to the field from the greenhouse, plants were inoculated with toothpick tip

inoculum. The lesion area was measured on the 21st day (07/11/03) after inoculation, and a 0-9 rating was used to evaluate SB development after an additional 1.5 months (08/6/03) (Groth et al., 1990; 1993). The plants were given a second inoculation on (08/6/03) to see the differences in SB development using the toothpick inoculation method in the collar of the last fully expanded leaf. The lesion produced from the second inoculation was measured after 7 days (08/13/03).

2.2.9 Extraction of Rice Genomic DNA from the Transgenic Plants

Three to five leaves from each transgenic plant and non-transgenic control plants were collected and used for PCR analysis. Rice genomic DNA from both transgenic and non-transgenic plants was isolated using the precipitation method (Sambrook et al., 1989). Two to 3g of rice leaf tissue was cut into small pieces, placed into a pestle where liquid nitrogen was added, so that the rice tissue was frozen, the tissue was ground into a powder and added to a 50mL plastic centrifuge tube. Three mL DNA extraction buffer (100mM Tris-HCl pH8.5, 100mM NaCl, 50mM EDTA pH8.0 and 2% SDS) was added to the centrifuge tube, mixed well and incubated in a 65C water bath for 1 hour. A solution consisting of equal volumes of phenol and chloroform was added to the centrifuge tube, mixed gently, and centrifuged for 5 minutes. The supernatant was transferred to a new tube, the same volume of isopropanol was added to the tube, the tube was maintained for 30 minutes at 4C, and the suspension was centrifuged for 10 minutes at 34,000rpm. The supernatant was again discarded and the pellet was washed with 70% ethanol and dried in a fume hood for 20 minutes. Two ml of sterile water was added to the tube to dissolve the pellet, 10uL RNaseA (10mg/uL) was added to the dissolved pellet, and the centrifuge tube was placed in a 37C water bath for 30 minutes. An equal volume of phenol/chloroform was then added, mixed gently, and the tube contents were centrifuged 5

minutes at 34,000rpm. The supernatant was transferred to a new tube where 3M NaAC, at one tenth volume of the supernatant, and 100% ethanol at 2.5 volume of the supernatant were added to the supernatant. The centrifuge tube was stored at -20C for 30 minutes, and then centrifuged for 10 minutes at 34,000rpm. The supernatant was discarded, the pellet was washed with 70% ethanol, and the pellet was dried in a fume hood overnight at room temperature. The DNA pellet was then dissolved in 500uL sterile water. Re-extraction was performed for each sample, using the same procedure, to obtain high quality DNA. DNA concentrations were determined by OD value at 260nm wave length using a Beckman Du-64 spectrometer. The formula used was: DNA (ng/ul) = $OD_{260} \times 50 \times \text{dilution factor}$.

2.2.10 Using PCR to Determine Whether the *bar* Gene Was Present in Transgenic Plants

Primers for the *bar* gene were designed in Dr. D. Shih's laboratory using the computer software Primer Select. The forward primer was 5'- TACCATGAGCCCAGAACGA-3', the reverse primer was 5'-TCAGATCTCGGTGACGGGCA-3', and the amplification size was 600bp. The primers were diluted to 10uM/uL.

The master mix was prepared as follows: 90uL (15x6, 15ml for each sample and 6 samples) sterile water was added to a sterilized microcentrifuge tube, 15uL (2.5x6) PCR buffer was added to the tube, 18uL (3x6) MgCl₂ was added to the tube, and 6uL (1x6) dNTP with 100nM concentration was added to the tube to become the master mix. The master mix was stirred well and divided into six PCR tubes, each tube had 21.5uL of the master mix. One uL of forward and 1uL of reverse primers were added to each tube. Three uL of non-transgenic rice genomic DNA was added to one tube which contained the master mix. Three uL rice genomic DNA from each of the transgenic plants 9-2, 46-1, 33-4, 15-7 and 33-3 was added to 5 different

tubes which contained master mix. The 6 PCR tubes were placed into adjacent wells in the thermalcycler (TECHNE, UK). Using hot start, 0.5uL Taq polymerase was added to each tube.

The PCR thermalcycler program was used for the *bar* gene as follows: one step at 94C for 5 minutes, 16 cycles at 94C for 1 minute each, 72 C (decreasing 1 C/cycle) for 1 minute, 72C for 1 minute, 28 cycles with 94C for 1 minute, 56C for 1 minute, 72C for 1 minute, and the final step at 72C for 10 minutes and hold at 4C.

2.2.11 Using PCR to Determine Whether the β -1, 3-glucanase and Chitinase Genes Were Present in Transgenic Plants

Primers for the β -1, 3-glucanase gene were designed in Dr. Shih's lab using the computer software Primer Select. The forward primer was 5'-TGCAAGATGGTGGGTACAGAAAAA T-3', the reverse primer was 5'-CTCGAGGGCAGCATACACAGAATC-3' and the amplification size was 476bp.

Master mix and the 6 DNA samples were the same as used for PCR of the *bar* gene, but the two primers were for the β -1, 3-glucanase gene. Using hot start, 0.5uL Taq polymerase was added to each tube.

The same PCR thermalcycler program was used for both the β -1, 3-glucanase and chitinase genes. One step at 94C for 5 minutes, 4 cycles with 94C for 1 minute, 72 C (decreasing 1 C/cycle) for 1 minute, 72 C for 1 minute, 35 cycles at 94C for 1 minute, 68C for 1 minute, 72C for 1 minute, and the final step at 72C for 10 minutes, then hold at 4C.

PCR primers to detect the chitinase gene were designed in Dr. D. Shih's lab using the computer software Primer Select. The forward primer was 5'-AGTGTGGAAGGCAAGC AGGACCTC-3', the reverse primer was 5'-CCAGGGGCGCAGGGGAAGT-3' and the amplification size was 457bp.

Master mix for the 6 DNA samples was the same as used for PCR of the bar gene, but the two primers were for the chitinase gene. Using hot start, 0.5uL Taq polymerase was added in each tube.

2.2.12 Southern Blot Test for Detecting β -1, 3-glucanase and Chitinase Genes in Transgenic Plants

Southern blot tests were conducted to confirm the PCR identification of the beta glucanase and chitinase genes in transformed plants. Plants were selected for testing based on their SB resistance level, which was based on each plants ratio of lesion length to plant height at the June 30, 2004 rating in the field. DNA was extracted from non-transgenic Taipei 309 (control), transgenic plants in group 1 (plants numbered 9-2, 33-3, 46-1, 45-2), group 2 (plant 33-4), and group 3 (plant 15-7).

Rice genomic DNA extracted from transgenic and non-transgenic plants as described in this chapter section 2.2.9 was digested with the restriction enzyme Hind III (50U/uL). Each 20ug sample of rice genomic DNA was digested with the Hind III. The digested DNA along with markers and controls were electrophoresed on an 0.8% agarose gel in 0.5x Tris-Borate-EDTA (TBE) buffer, and the gel was run at 50 V constant for 5 hours or until the bromophenol blue dye almost ran out of the gel. One kb DNA mass ladder and λ marker (Gibco BRL Life Technologies) were used as markers. Controls included 0.5ng PCR products amplified from β -1, 3-glucanase and chitinase. The gel was stained with ethidium bromide (5ug/ml) for 20 minutes, destained for 5 minutes in 100ml water, and photographed with a fluorescent ruler on an UV-transilluminator. The gel was place in 100ml 0.25N HCl for 10 minutes with gentle shaking so that large DNA fragments could be nicked for efficient transfer. The gel was washed in 100ml water briefly and soaked in transfer buffer (1 M NaCl, 0.4 M NaOH) twice for 15 minutes each time. The DNA in the gel was transferred to Zeta-probe-GT (genomic tested) nylon

membrane (BioRadTM) by downward capillary alkaline transfer overnight. The DNA was crosslinked to the membrane by UV-irradiation in a Stratalinker crosslinker (Stratagene) on auto setting delivering 1.2×10^5 ujoules of energy. The membrane was washed briefly in 100mL water, dried and pre- hybridized with 9ml ($0.1\text{mL}/\text{cm}^2$) DIG Easy Hyb buffer plus 20uL salmon sperm DNA for 1 hour at 43°C in a roller bottle.

Fifty microliters of the ³²P labeled DNA probe (see below) was boiled with 80uL salmon sperm DNA for 10 minutes. The probe mixture was kept on ice and added to a roller bottle with 9mL Ultrahyb Hybridization SolutionTM (Ambion) to hybridize at 43C overnight. The hybridization solution was discarded using proper radioactive waste disposal. The membrane was washed once with 30ml low stringency wash buffer (2X SSC, 0.1%SDS) at room temperature for 10 minutes. The membrane was washed twice with 30ml high stringency buffer (0.1X SSC, 0.1%SDS) for 30 minutes each time while maintained at 43C. The membrane was air dried, wrapped with plastic membrane and hybridization bands were detected with the Storm phosphoimager 860TM (Molecular Dynamics).

The probes were synthesized using PCR products from plasmids containing β -1, 3-glucanase or chitinase gene as templates (Khan, 2002). PCR master mix had 60uL sterilized water, 10uL PCR buffer, 12uL MgCl, and 4uL dNTP. The master mix was divided into 4 tubes each tube had 21.5uL master mix, two tubes for β -1, 3-glucanase gene PCR, and two tubes for chitinase gene PCR.

For PCR of the β -1, 3-glucanase gene, a 2uL plasmid solution of the β -1, 3-glucanase gene, 2uL forward primer with sequence 5'-ATGGCTGCTATCACACTC-3', and 2uL reverse primer with sequence 5'-ACCTCACATCTCCTTACGA-3' were mixed together, then each half

of the mixture was added to different tubes containing 21.5uL master mix. The size of the amplification product was approximate one kb.

For PCR of the chitinase gene, a 2uL solution of the plasmid containing the chitinase gene, 2uL forward primer with sequence 5'- GATGATTATGATATGCAGTFTA-3' and 2uL reverse primer with sequence 5'-ATGAAGFCATCGTAGGTGTA-3' were mixed together as in the previous mixture. Half of the mixture was added to each of two tubes with 21.5uL master mix. The amplification size was approximate one kb.

This PCR thermalcycler program was used for both genes: one step at 94C for 5 minutes, 6 cycles with 94C for 1 minute, 58 C (decreasing 1 C/cycle) for 1 minute, 72 C for 1 minute, 28 cycles with 94C for 1 minute, 52C for 1 minute, 72C for 1 minute, and the final step at 72C for 10 minutes. Using hot start, 0.5uL Taq polymerase was added to each tube.

The PCR products were purified by NucAwayTM spin columns (Ambion). The final concentration of β -1, 3-glucanase gene was 65ng/uL and the final concentration of the chitinase gene was 125ng/uL based on the OD value at 260nm wave length on a Beckman Du-64 spectrometer.

The PCR product of β -1, 3-glucanase gene was diluted 5 times and the chitinase gene product was diluted 10 times using sterile, deionized water to about 12.5ng/uL for both genes. Two uL (25ng) of each diluted PCR product was added to two tubes with 21uL sterile deionized water. The two tubes were boiled for 5 minutes, cooled immediately on ice, and each tube had the following components added: 15uL of random primers from Promega Company, 2uL dATP, 2uL dGTP, 2uL dTTP and 1uL Klenew DNA polymerase from Promega Company. The solution in each tube was mixed thoroughly, and 5uL [³²P]-labeled dCTP was added to each tube, and the solution was allowed to stand at room temperature for 2 hours to make labeled probes. Each tube

had 50uL probe at 0.5ng/uL. The probes were purified using NucAway™ spin columns, 2uL probe was added to 10ml of scintillation fluid, and the probe activity was calculated (2.63×10^5 CPM/uL for both probes). Forty-eight uL of probe was used so that the total radioactivity was 1.37×10^7 CPM for the hybridization.

2.3 RESULTS

2.3.1 Transformation and Regeneration

The transformation procedure was conducted over a 2-day period. The first set of transformation biolistics produced 14 dishes of putatively transformed calli. The second day of biolistic transformations produced 20 dishes of putatively transformed calli. A total of 104 plants were regenerated from the first set of calli, and 159 plants were regenerated from the second set of treated calli. One hundred and one transgenic plants were transplanted into the field at the Rice Research Station, Crowley, LA.

2.3.2 Field Tests

2.3.2.1 Tests for Hygromycin B Resistance

One hundred transgenic plants were tested for resistance to hygromycin B. Cluster analysis (SAS/STAT User's Guide, 1994) was used to group transgenic plants into three groups according to lesion length. Group 1 had no lesions formed in response to treatment with hygromycin B. Group 2 had lesions from 1-3mm in length and group 3 had lesions greater than 3mm in length (Table 2.1). Group 1 plants were not different from the hygromycin resistant control plants. Groups 2 and 3 had lesions significantly longer than the group 1 and resistant control plants. Groups 1 and 2 had significantly shorter lesions than the non-transgenic control plants and group 3 plants had significantly longer lesions, suggesting that they were more susceptible than the susceptible control plants (Table 2.1). The percentage of transgenic and

non-transgenic plants in each group is shown in Figure 2.14. There were no non-transgenic plants in group 1. Transgenic plants in group 2 had significantly shorter lesions than the non-transgenic control plants and longer lesions than the resistant control. This suggested that the transgenic plants in group 1, with no lesions produced, were resistant. A resistant lesion typical of those produced on the transgenic (resistant) control is shown in Figure 2.15.

A resistant reaction by the transgenic resistant control is shown in Figure 2.15. A susceptible reaction, typical of the non-transgenic control plants, is shown in Figure 2.16. Necrotic lesion length on susceptible plants was significantly correlated with the extended lesion length with a Pearson correlation coefficient of 0.66 and P-value<0.0001.

When 100 transgenic plants were tested in the field with hygromycin B, 79 were resistant with no lesions produced and 21 were susceptible with lesions or extended lesions produced in reaction to hygromycin.

Table 2.1. Multiple comparisons of each susceptibility group, based on mean lesion length, against resistant and non-transgenic control plants for hygromycin B test in the field.

Susceptibility group	Lesion length range (mm)	Number of plants	Mean lesion length (mm)	Standard error
1	0	79	0.0a ^x	0.000
2	1 – 3	14	1.9ab ^y	0.199
3	4 – 10	7	4.8ab	0.409
Resistant Control		8	0.0	0.289
Non-transgenic Control		10	3.0	0.259

^x significant at 0.05 level compared with non-transgenic control.

^y significant at 0.05 level compared with resistant control.

Dunnett's t Tests for lesion length.

Note: This test controls the Type I experimentwise error for comparisons of all groups against each control.

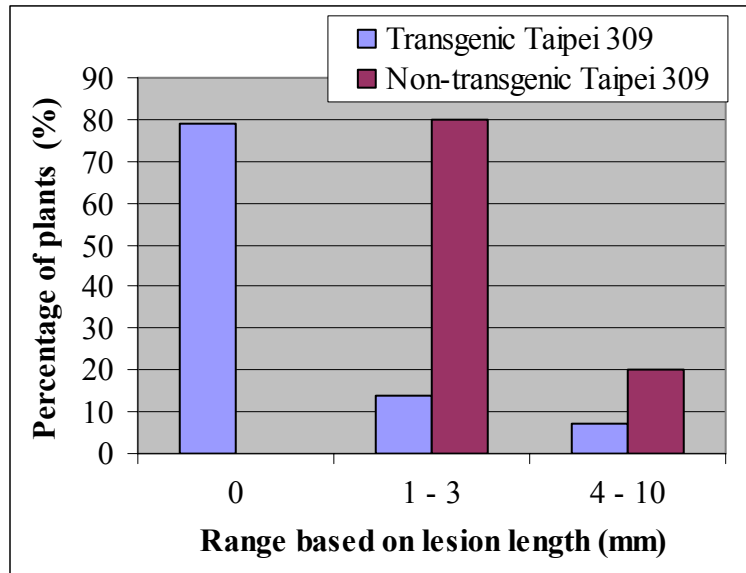


Figure 2.14. Percentage of plants placed into each susceptibility group using cluster analysis based on mean lesion length after exposure to hygromycin B in a field test.



Figure 2.15. Reaction of resistant transgenic control plant after exposure to hygromycin B.



Figure 2.16. Susceptible reaction of susceptible non-transgenic control plant to hygromycin B.

2.3.2.2 Demonstration of Liberty Resistance and Presence of the *bar* Gene in Transgenic Plants

2.3.2.2.1 Evaluation of Transgenic Plants for Liberty Herbicide Resistance

Ninety nine transgenic plants were tested for resistance to Liberty herbicide by exposure to Liberty at 363ppm ai. All of the resistant control plants produced no lesions in response to Liberty.

Eight of 99 transgenic plants produced no lesion in response to Liberty. Five out of the 8 resistant plants (21-2, 41-1, 41-2, 44-1, 46-1) had only scattered brown spots on each tested leaf, 3 of the 8 resistant plants (18-1, 31-9, 33-1) had slight browning on the leaf edges. Seven of the 8 plants resistant to Liberty herbicide were also resistant to hygromycin B. Plant 33-1 was not resistant to hygromycin B. The gene may have been present but not expressing. Figure 2.17 shows the resistant reaction with no lesion, but some browning on the leaf edge. Figure 2.18 shows a similar resistance reaction on plants exposed to Liberty herbicide in a greenhouse test. Figure 2.19 shows a typical susceptible reaction with a solid necrotic lesion and extended yellowing or browning of the edge of the treated blade.

The eight transgenic plants without lesions were placed into group 1 by cluster analysis. The 91 transgenic plants that had different levels of lesion development were grouped into three groups based on cluster analysis (SAS/STAT User's Guide, 1994) (Table 2.2). Plants in groups 1 and 2 had significantly less lesion development than the susceptible non-transgenic control plants, but plants in group 4 had significant longer lesions than the non-transgenic control. Plants in group 3 were not significantly different from the non-transgenic control. Plants in group 1 were not significantly different than the resistant control. But plants in group 2, group 3 and group 4 had significantly longer lesion than the resistant control.



Figure 2.17. Resistant reaction on transgenic plant treated with Liberty herbicide in the field. One or two black pen stripes indicate exposure to 363ppm or 750ppm Liberty, respectively.



Figure 2.18. Resistant reaction to Liberty herbicide on transgenic plants grown in the greenhouse. Two stripes indicate exposure to 750ppm Liberty.



Figure 2.19. Susceptible reaction Liberty herbicide on non-transgenic plants in the field. One and two stripes indicate 363ppm and 750ppm Liberty, respectively.

As all resistant control plants showed no lesions, higher resistance levels could not be detected by comparing with the resistant control. But by comparing the resistance reaction of all plants with the susceptible non-transgenic control, it appears that some transgenic plants in group 2 may have resistance at a lower level than the resistant control. The percentage of plants in each susceptibility group is shown in Figure 2.20. There were no non-transgenic plants in group 1.

The extended lesion on each tested plant was also analyzed to confirm the resistance level. Five transgenic plants (plants # 21-2, 41-1, 41-2, 44-1, 46-1) with no lesions also had no extended lesions and were grouped together. The rest of the transgenic plants were in different groups based on cluster analysis (SAS/STAT User's Guide, 1994) (Table 2.3).

Compared with non-transgenic control plants, transgenic plants in group 1, group 2 and group 3 showed significantly less extended lesion length, but group 4 had no difference. Compared with the resistant control plants, transgenic plants in group 1 were not significantly different, but plants in groups 2, 3 and 4 had significantly longer extended lesion lengths. Combining results from both controls, some moderately resistant plants may be in group 2 or

group 3, but low levels of resistance could not be detected. Again, there were no non-transgenic plants in group 1 (Figure 2.21).

Table 2.2. Multiple comparisons of each susceptibility group, based on mean lesion length, against resistant and susceptible non-transgenic control plants for Liberty herbicide (363ppm).

Susceptibility group	Lesion length range (mm)	Number of plants	Mean lesion length (mm)	Standard error
1	0	8	0.0a ^x	0.000
2	5-30	60	18.0ab	1.530
3	35-62	21	46.3b ^y	2.586
4	70-140	10	89.7ab	3.747
Resistant Control		8	0.0	4.190
Non-transgenic Control		13	50.3	3.287

^x significant at 0.05 level compared with non-transgenic control.

^y significant at 0.05 level compared with resistant control.

Dunnett's t Tests for lesion length.

Note: This test controls the Type I experimentwise error for comparisons of all groups against each control.

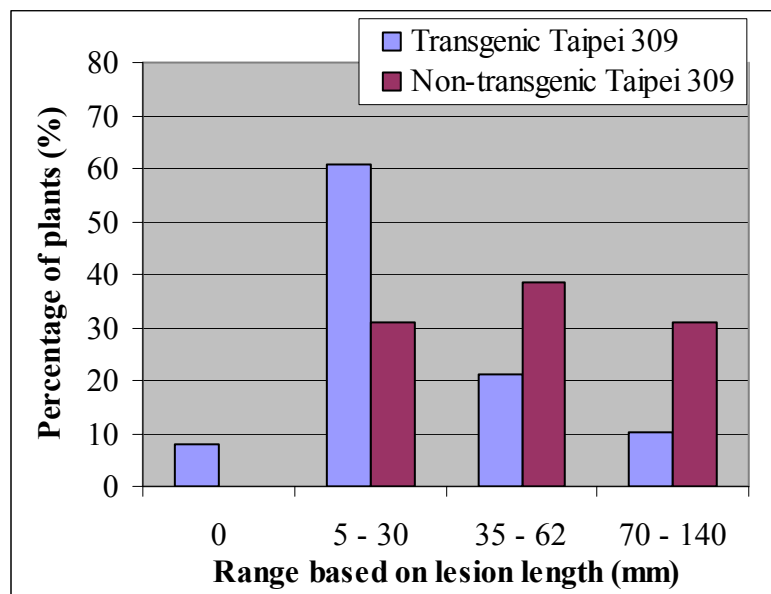


Figure 2.20. The percentage of transgenic and non-transgenic Taipei 309 plants in each lesion class, established by cluster analysis, after treatment with Liberty herbicide (363ppm).

Table 2.3. Multiple comparisons of each susceptibility group, based on the mean extended lesion length, with the resistant and non-transgenic control plants for reaction to Liberty herbicide (363ppm).

Susceptibility group	Extended lesion range (mm)	Number of plants	Mean extended lesion length (mm)	Standard error
1	0	5	0.0a ^x	0.000
2	36- 95	16	77.3ab	5.460
3	98-145	45	117.7ab	3.256
4	148-230	33	178.2b ^y	3.802
Resistant Control		8	18.4	6.057
Non-transgenic Control		13	171.5	7.721

^x significant at 0.05 level compared with non-transgenic control.

^y significant at 0.05 level compared with resistant control.

Dunnett's t Tests for extended lesion length.

Note: This test controls the Type I experimentwise error for comparisons of all susceptibility groups against each control.

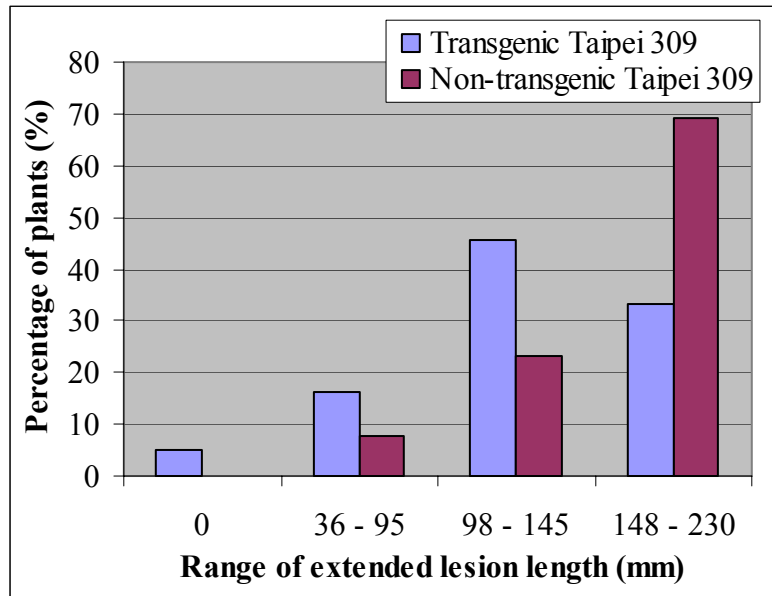


Figure 2.21. The percentage of transgenic and non-transgenic Taipei 309 plants in each extended lesion susceptibility group based on cluster analysis on plants tested against Liberty herbicide (363ppm).

The Pearson correlation coefficient for lesion length vs extended lesion length was 0.53277 (P-value < 0.0001). This indicated that combined lesion and extended lesion length could be used to determine the resistance of tested plants to Liberty herbicide. When lesions and extended lesions were both zero this indicated a strongly resistant reaction. Three transgenic plants (18-1, 31-9 and 33-1) with no necrotic lesion, but with extended lesion lengths of 130mm (group 2), 80mm (group 1) and 95mm (group 1) may have had an intermediate level of resistance. When no necrotic lesion was formed, but the extended lesion was not zero, the resistance level may have been at some intermediate level, thereby offering only partial resistance to Liberty herbicide. Thus, to analyze extended lesion length for transgenic plants with no necrotic lesions could give some information on moderate Liberty resistance.

Four transgenic plants (41-1, 41-2, 44-1, and 46-1) that produced no necrotic lesions and had no extended lesions in reaction to Liberty herbicide at 363ppm were also tested with 750ppm Liberty. The symptoms produced were the same at both concentrations. This indicated that strongly resistant transgenic plants were resistant at all Liberty concentrations tested. Less resistant plants reacted differently to the two Liberty concentrations. Forty three transgenic plants were used for analyzing the effects of Liberty herbicide at 363ppm and 750ppm. The lesion lengths produced at 363ppm and 750ppm were significantly different, as were the extended lesion lengths (Table 2.4). So with moderate resistance, different concentrations of Liberty could produce significantly different reactions.

2.3.2.2.2 Proving the Presence of the *bar* Gene in Transgenic Plants Using the PCR Method

Using cluster analysis grouping based on lesion length, DNA was extracted from non-transgenic Taipei 309, a transgenic plant from group 1 (46-1) with strong resistance, two plants from group 2 (9-2, 15-7), and one plant from group 3 (33-3). DNA was also extracted

from a plant (33-4) that was not tested with Liberty herbicide, but had a high level of sheath blight resistance.

The results indicated that not all of the transgenic plants had the *bar* gene (Figure 2.22). The extra bands on lanes 3, 4 and 6 were non-specific amplification. Transgenic plant # 46-1 (lane 4), with strong resistant to Liberty herbicide (no necrotic lesion or extended lesion), was transformed with the *bar* gene. Transgenic plant# 15-7 (lane 6) in group 2 had the *bar* gene, but also had a 25mm long lesion, whereas, another transgenic plant# 9-2 (lane 3) did not have the *bar* gene, but produced a 30mm lesion. This was why moderate resistant could not be easily detected in the evaluation process. Transgenic plant # 33-3 (lane 7) had the *bar* gene, but this plant's resistant level was very low with a 40mm lesion and 125mm extended lesion. This may indicate that it's *bar* gene was silenced as this was a lesion typical of a susceptible, non-transgenic plant.

Table 2.4. Paired t test for necrotic and extended mean lesion length when transgenic Taipei 309 plants were tested with 750ppm and 363ppm of Liberty herbicide.

	Mean difference between necrotic and extended lesion lengths when tested with 750ppm and 363ppm liberty	t	P-value
Necrotic lesions	15.35 ^a	3.3585	0.0017
Extended lesion	32.95 ^b	6.7178	0.0001

^a Necrotic mean lesion lengths of 363ppm and 750ppm were significant different at the 0.01 level.

^b Extended mean lesion length of 363ppm and 750ppm were significant different at the 0.01 level.

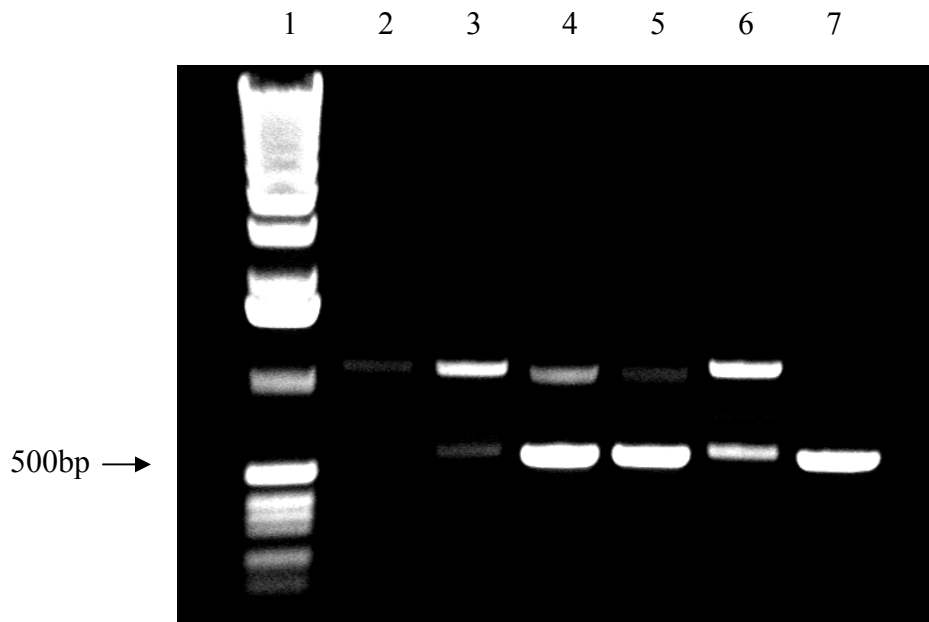


Figure 2-22. PCR analysis for *bar* gene in transgenic and non-transgenic Taipei 309 plants. Lane 1: 1kb molecular marker; lane 2: non-transgenic plant; lane 3: transgenic plant# 9-2 from group 2; lane 4: transgenic plant# 46-1 from group1; lane 5: transgenic plant# 33-4 which was not tested for resistance to Liberty herbicide, but was known to have a high level of sheath blight resistance; lane 6: transgenic plant# 15-7 from group 2; lane 7: transgenic plant# 33-3 from group 3.

2.3.2.3. Evaluation for Sheath Blight Resistance

2.3.2.3.1 First Inoculation of Transgenic Plants in the Field

The height of transgenic plants averaged 548mm and non-transgenic plants averaged 572mm in height. The mean height difference was not significant with a t value of 0.87 (P value=0.3892).

Seventy nine transgenic plants were successfully inoculated with *R. solani* in the first field inoculation. The lesion lengths and plant heights were measured for each plant. Using cluster analysis on the ratio of lesion length to plant height, transgenic plants were divided into four groups. The transgenic plants in group 1 (Figures 2.23 and 2.24) had significantly less

disease than non-transgenic plants (Figure 2.25) (Table 2.5). Some transgenic plants were in group 4 and had significantly more disease than non-transgenic control plants. All non-transgenic plants were in groups 2 and 3 (Figure 2.27). The susceptible Cocodrie control plants (Figure 2.26) were also in group 2, group 3 and group 4 (Figure 2.27). Transgenic plants in group 4 were not significantly different from Cocodrie. Non-transgenic TP309 and transgenic TP309 plants in groups 1, 2, and 3 showed significantly different ratios of lesion length to plant height.



Figure 2.23. Transgenic Taipei 309 plant showing a high level of sheath blight resistance 3 weeks after inoculation in the field and placed by cluster analysis in group 1 (resistant plants).



Figure 2.24. Transgenic Taipei 309 plant showing a high level of sheath blight resistance 3 weeks after inoculation with *Rhizoctonia solani* in a field test. Cluster analysis placed this plant in group 1 (resistant).



Figure 2.25. Non-transgenic Taipei 309 plant 3 weeks after inoculation with *Rhizoctonia solani* in the field showing a susceptible reaction and placed by cluster analysis of lesion size in group 3 (susceptible).

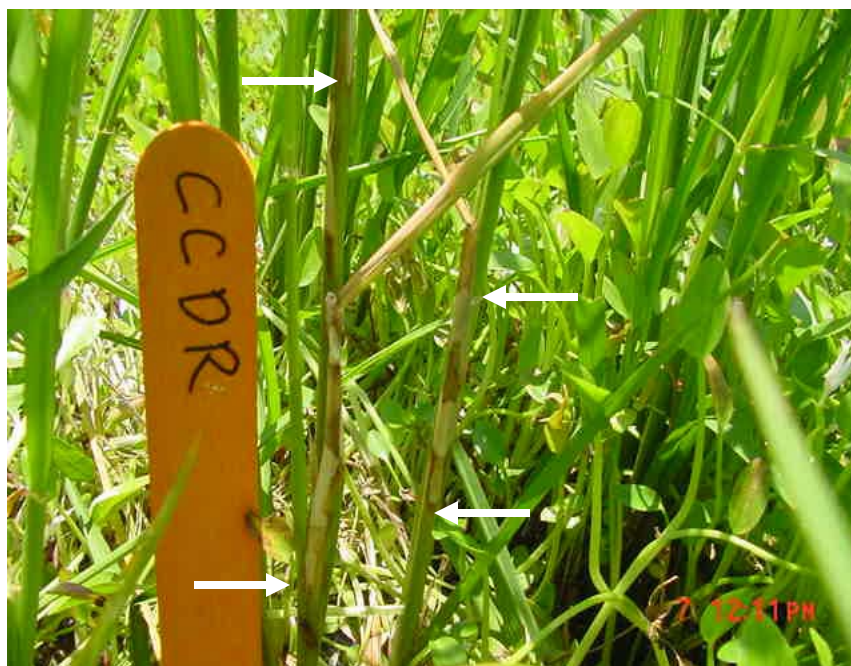


Figure 2.26. Susceptible Cocodrie control plant showing a very susceptible sheath blight reaction 3 weeks after inoculation with *Rhizoctonia solani* in a field test. Cluster analysis of lesion length placed this plant in group 4 (very susceptible).

Table 2.5. Multiple comparisons of each susceptibility group, based on the mean ratio of lesion length to plant height, against non-transgenic Taipei 309 and Cocodrie after the first field inoculation.

Susceptibility group	Ratio range of lesion length /plant height	Number of plants	Mean of lesion length /plant height	Standard error
1	0 – 9.23	26	3.8a ^x b	1.1508
2	9.24 - 20.34	37	15.1b ^y	0.9647
3	23.44 - 33.33	11	28.8ab	1.7692
4	42.11 - 68.75	5	52.4a	2.6241
Non-transgenic Taipei 309		14	16.3b	1.5682
Cocodrie control		14	25.6a	1.5682

^x significant at 0.05 level compared with non-transgenic control.

^y significant at 0.05 level compared with Cocodrie control.

Dunnett's t Tests for ratio of lesion to plant height.

Note: This test controls the Type I experimentwise error for comparisons of all susceptibility groups against each control.

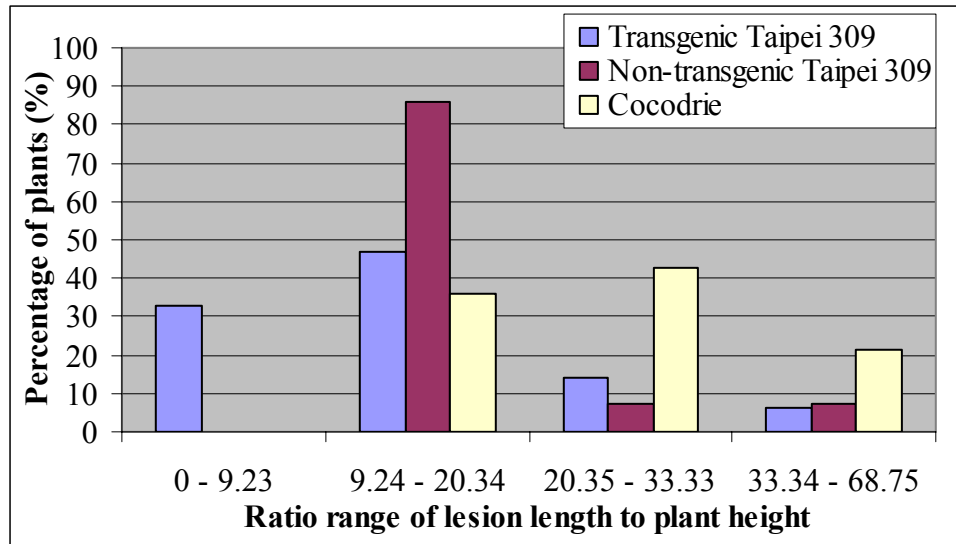


Figure 2.27. The percentage of plants in each ratio range, for lesion length/plant height, including transgenic and non-transgenic TP309 and the susceptible Cocodrie control after the first inoculation. Ranges were calculated by cluster analysis.

Based on lesion length, transgenic plants were clustered into 4 groups. The transgenic plants in the first group had significant less disease than non-transgenic plants. Transgenic plants in groups 2 and 3 did not show significantly less disease than non-transgenic plants, but transgenic plants in group 4 had significantly more disease than non-transgenic TP309.

Compared with the susceptible Cocodrie control plants, non-transgenic TP309 and transgenic TP309 in groups, 1, 2 and 3 had significantly less sheath blight. Transgenic plants in group 4 were not significantly different for sheath blight than Cocodrie (Table 2.6, Figure 2.28).

The transgenic plants grouped in a similar way whether the grouping was based on lesion length/plant height ratio or on lesion length. Sixteen transgenic plants (10, 9-2, 12-2, 13-1, 14-1, 15-4, 18-3, 19-1, 24-1, 25-1, 39-1, 32-1, 33-3, 39-1, 45-2, and 46-1) were in group 1, 14 transgenic plants (9-1, 11-2, 12-1, 15-3, 15-8, 18-1, 20-4, 21-2, 23-1, 25-5, 31-1, 31-6, 31-8, and 44-1) were in group 2, four transgenic plants (6-3, 18-4, 20-5, and 29-2) were in group 3, and

five transgenic plants (7-1, 7-3, 20-3, 33-5, and 36-2,) were placed in group 4 with both methods.

The resistant transgenic plants in group 1 were detected by both methods.

Table 2.6. Multiple comparisons of each susceptibility group based on mean lesion length when compared to non-transgenic and Cocodrie control plants after the first inoculation.

Susceptibility group	Range of lesion length (mm)	Number of plants	Mean lesion length (mm)	Standard error
1	0 - 10	17	4.7a ^x b	6.5117
2	15 - 36	24	24.7b	5.4804
3	40 - 60	23	50.2b	5.5983
4	70 - 220	15	104.7a	6.9322
Non-transgenic Taipei 309		14	38.4b	7.1755
Cocodrie		14	93.9a	7.1755

^x Compared with non-transgenic plants showed significant different at the 0.05 level.

^y Compared with susceptible Cocodrie plants showed significant difference at the 0.05 level.

NOTE: This test controls the Type I experimentwise error for comparisons of all susceptibility group against a control.

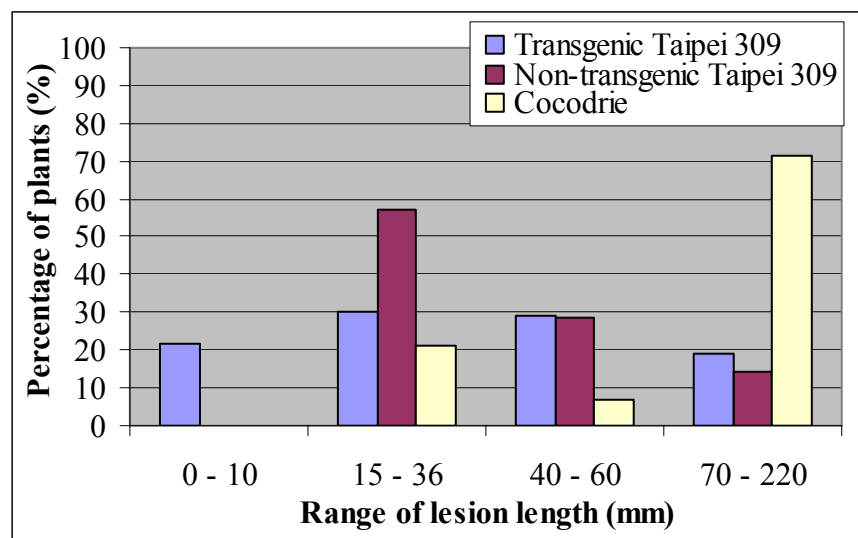


Figure 2.28. The percentage of plants in each range of lesion length for transgenic and non-transgenic Taipei 309 and Cocodrie after the first inoculation. Ranges were calculated by cluster analysis.

There were slight differences in grouping the transgenic plants using the two methods, but only in groups 2, 3, and 4. Transgenic plant 33-4 was in group 2 based on ratio, but in group 1 based on lesion length. Ten transgenic plants were in group 1 based on ratio, but in group 2 based on lesion length (3, 6-2, 11-1, 18-7, 25-3, 31-5, 31-7, 33-1, 40-2, and 41-1). Nineteen transgenic plants were in group 2 based on ratio, but in group 3 based on lesion length (8, 7-5, 14-2, 15-5, 15-6, 18-2, 18-5, 20-1, 22-1, 22-2, 25-2, 25-4, 31-4, 33-2, 36-1, 37-1, 42-1, 42-2, and 45-1). Three transgenic plants were in group 2 based on ratio, but in group 4 based on lesion length (19-2, 20-2, and 41-2). Seven transgenic plants were in group 3 based on ratio, but in group 4 based on lesion length (40-1, 28-1, 22-3, 24-2, 15-7, 18-6, and 6-1).

Non-transgenic control plants were only in groups 2 (85.7%), 3 (14.0%) and 4 (14.0%) using ratio, but 57.1%, 28.6% and 14.3% using lesion length. Thus, more non-transgenic TP309 plants were in group 2 using ratio than using lesion length. Susceptible Cocodrie plants were also in groups 2 (35.7%), 3 (42.9%) and 4 (21.4%) using ratio, but 21.3%, 7.1% and 71.4% using lesion length. Cocodrie plants were clearly more susceptible than Taipei 309 plants and lesion length appeared to give a better measure of susceptibility than lesion length/plant height ratio.

The 0-9 disease rating on control and transgenic plants for sheath blight at maturity (Figures 2.29, 2.30 and 2.31) was also analyzed. Transgenic plants in group 1 had significantly less disease than the controls. Group 3 plants had significantly more disease than the non-transgenic control plants (Table 2.7). There were no non-transgenic plants in group 1 (Figure 2.32). The ratio of lesion length/plant height measured 3 weeks after inoculation was significantly correlated with the 0-9 rating taken right before maturity, with a Pearson correlation coefficient of 0.35297 (P value = 0.001). The lesion length measurement was also correlated with the 0-9 rating, with a Person correlation coefficient of 0.37640 (P value = 0.0004). This

suggested that the early measurements of disease reflected disease development during the rest of the season.

2.3.2.3.2 Sheath Blight Data Collected after Second Inoculation in the Field

A second inoculation was made at the heading stage of growth of the transgenic plants in the field and lesion length was measured for each transgenic and non-transgenic control plant. As all Cocodrie plants had very severe sheath blight disease, with lesions to the flag leaf sheath, it was impossible to inoculate these plants a second time. The height of transgenic plants averaged 572mm and non-transgenic plants averaged 548mm. The difference was not significant with a t-value of 0.87 (P value = 0.39).

Based on cluster analysis of lesion lengths, transgenic plants were placed into 3 groups. The multiple comparison analysis is shown in Table 2.8 and Figure 2.33.



Figure 2.29. Transgenic Taipei 309 plant with a 0.5 rating on the 0-9 sheath blight scale at maturity. This plant was placed in group 1 based on cluster analysis of 0-9 ratings (highly resistant). This is the same plant shown at an earlier growth stage in Figure 2.23.



Figure 2.30. Transgenic Taipei 309 plant with a 2.0 rating on the 0-9 sheath blight rating scale at maturity. This plant was grouped by cluster analysis into group 2 (resistant). This plant was classified in group 2 when it's lesion was measured 3 weeks after inoculation.



Figure 2.31. Transgenic Taipei 309 plant with a 5.0 rating on the 0-9 sheath blight rating scale when rated at maturity. This plant was placed in group 3 based on cluster analysis of 0-9 ratings (susceptible).

Table 2.7. Multiple comparisons of susceptibility groups, based on mean 0-9 ratings, against the non-transgenic control at the end of the season.

Susceptibility group	Disease rating range	Number of plants	Mean rating (0-9)	Standard error
1	0 - 0.5	40	0.5a ^x b	0.1724
2	1 - 4	23	1.4ab	0.2677
3	>4	11	5.34ab	0.2677
Non-transgenic				
Taipei 309		14	2.6b ^y	0.2949
Cocodrie		14	6.9a	0.2949

^x Compared with non-transgenic plants showed significant different at the 0.05 level.

^y Compared with Cocodrie plants showed significant different at the 0.05 level. Based on Dunnett's t Tests for disease rating.

NOTE: This test controls the Type I experimentwise error for comparisons of all groups against a control.

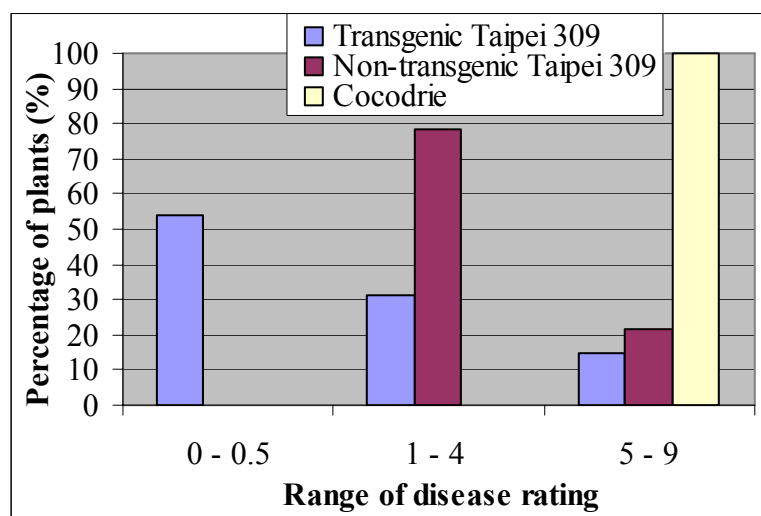


Figure 2.32. The percentage of plants in each 0-9 rating range for transgenic and non-transgenic Taipei 309 and Cocodrie at maturity.

Table 2.8. Multiple comparisons of each susceptibility group based on comparison of mean lesion lengths against those of the non-transgenic control plants following the second inoculation of plants in the field with *Rhizoctonia solani*.

Susceptibility group	Range of lesion lengths (mm)	Number of plants	Mean lesion length (mm)	Standard error
1	4 – 27	25	14.6a ^x	2.1553
2	30 – 50	24	41.0a	2.1997
3	55 – 80	28	65.3	2.0366
Non-transgenic control plants		9	63.6	3.5922

^x Compared with non-transgenic plants showed significant different at the 0.05 level based on Dunnett's t Tests for lesion length.

NOTE: This test controls the Type I experimentwise error for comparisons of all groups against a control.

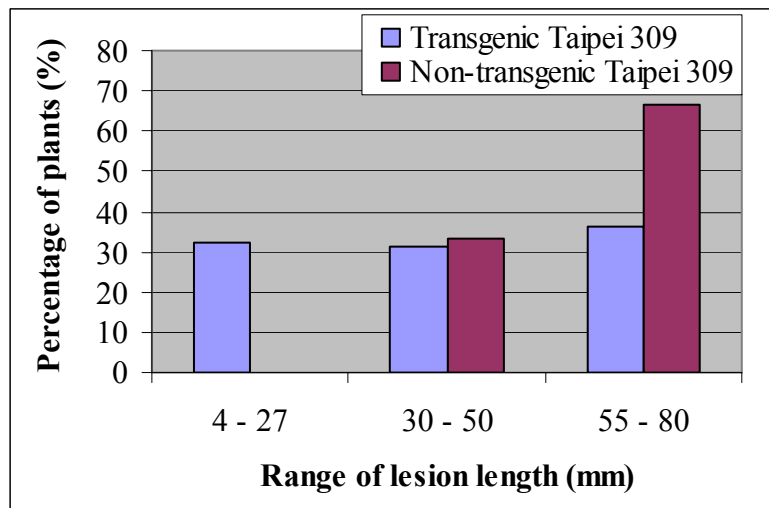


Figure 2.33. The percentage of plants in each group based on the lesions produced after the second inoculation with *Rhizoctonia solani* in the field.

As with the first inoculation, the transgenic plants inoculated a second time tended to group in a similar manner when the grouping was based lesion length. Twenty three transgenic plants were in group 1 (17-1, 18-2, 18-3, 18-5, 18-7, 20-1, 20-4, 21-3, 25-2, 28-1, 29-3, 31-7, 31-8, 33-2, 33-4, 33-5, 33-6, 36-2, 39-1, 40-1, 45-2, 6-1, and 7-2) based on lesion length.

The lesion length for the first inoculation was not correlated with lesion length in the second inoculation. The correlation coefficient was -0.052 (P value = 0.69). This may indicate that disease development slowed during the season, which was expected. Also, the environmental conditions were different late in the season with cooler, drier weather and sheath blight developed at a slower rate.

2.3.2.3.3 PCR and Southern Blot Analysis for β -1, 3-glucanase and Chitinase Genes

The PCR results indicated that tested transgenic plants had both the β -1, 3-glucanase and chitinase genes (Figure 2.34, Figure 2.36). Southern blot results confirmed that both genes were transferred to all the tested transgenic plants (Figure 2.35, Figure 2.37), even though the copy numbers of each gene were different.

Transgenic plants had 4 -5 copies of the β -1, 3-glucanase gene (Figure 2.35). The hybridization bands at different positions indicated that the transgenic plants were from different transformation events. Transgenic plants 9-2, 46-1 and 15-7 that had a strong PCR signal on lanes 3, 4 and 6 (Figure 2.34) also had a strong signal in the southern blot test on lanes 4, 5 and 7 (Figure 2.35). Transgenic plants 33-4 and 33-3 that had a weak PCR signal on lanes 5 and 7 (Figure 2.34) also had a weak signal in the southern blot test on lanes 6 and 8 (Figure 2.35).

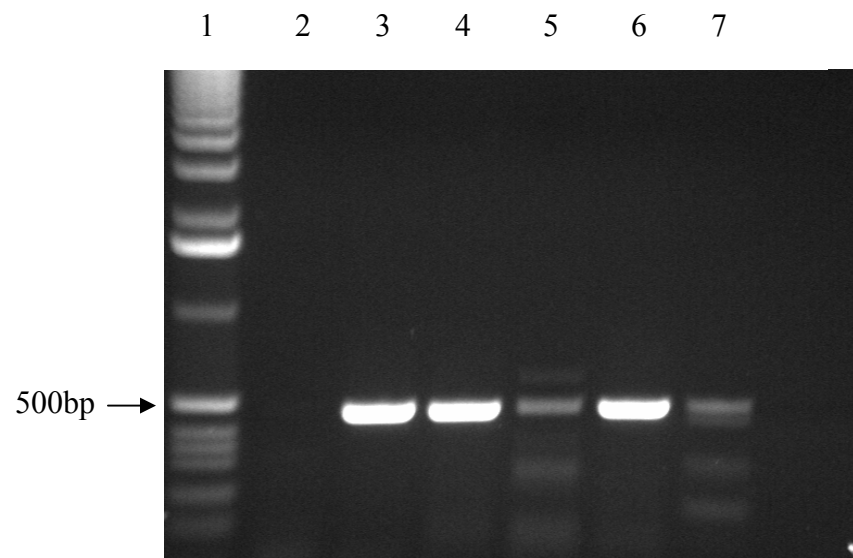


Figure 2.34. PCR analysis for β -1, 3-glucanase gene from transgenic and non-transgenic Taipei 309 plants. Lane1: 1kb marker; line 2: a non-transgenic plant. Lanes 3, 4 and 7: refer to transgenic plants 9-2, 46-1 and 33-3 from group 1. Lane 5: the transgenic plant 33-4 from group 2. Lane 6: the transgenic plant 15-7 from group 3.

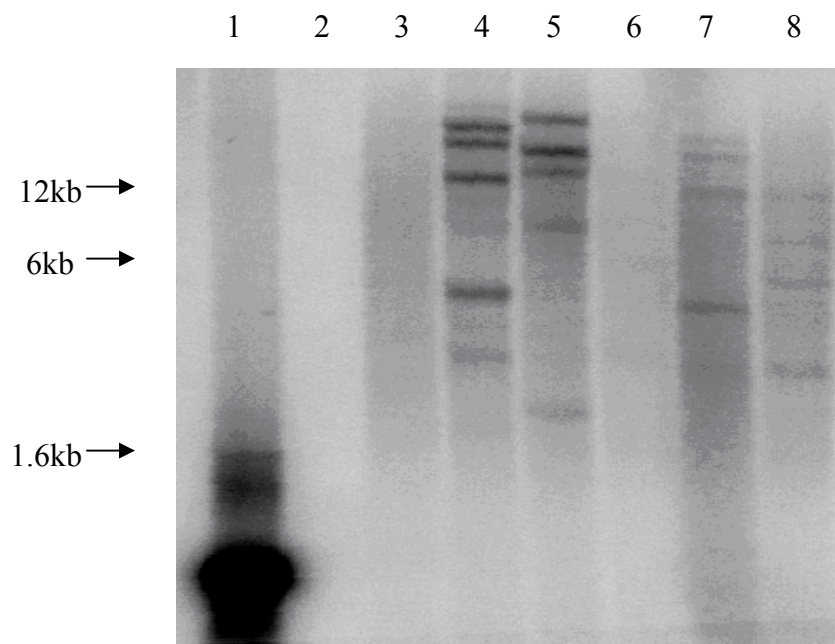


Figure 2.35. Southern blot analysis for β -1, 3-glucanase gene in transgenic and non-transgenic Taipei 309 plants. Lane 1 was positive control. Lane 2 was negative control. Lane 3 was a plant from non-transgenic control. Lanes 4, 5, and 8 were transgenic plants 9-2, 46-1 and 33-3 from group 1. Lane 6 was transgenic plant 33-4 from group 2. Lane 7 was transgenic plant 15-7 from group 3.

In the PCR test for chitinase detection, transgenic plants had the transgene (Figure 2.36). The hybridization bands at different positions further confirmed that the transgenic plants were from different transformation events (Figure 2.37). The two transgenic plants 9-2 and 46-1, lines 3 and 4, with the strongest PCR signals (Figure 2.36) also had the strongest southern blot signals on lanes 4 and 5 (Figure 2.37). Three other transgenic plants, 33-4, 15-7 and 33-3, with a strong PCR signal (Figure 2.35) also had strong southern blot signals Figure 2.37).

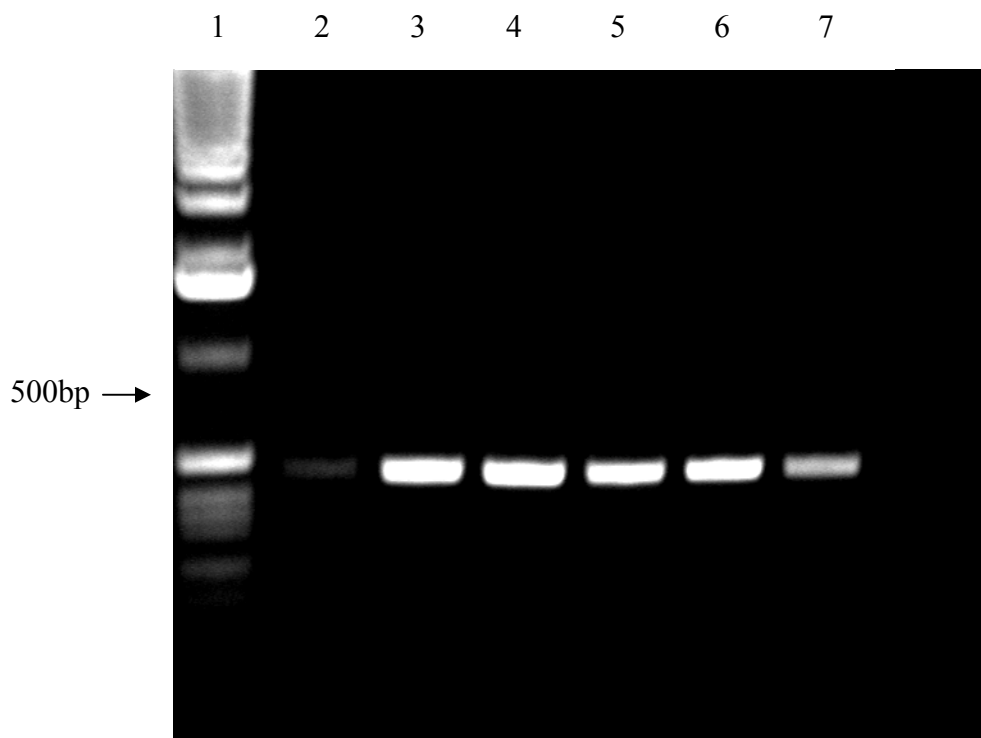


Figure 2.36. PCR analysis for the chitinase gene in transgenic and non-transgenic Taipei 309 plants. Lane1: 1kb marker; line 2: a non-transgenic plant. Lanes 3, 4, and 7: refer to transgenic plants 9-2, 46-1 and 33-3 from group 1. Lane 5: the transgenic plant 33-4 from group 2. Lane 6: the transgenic plant 15-7 from group 3.

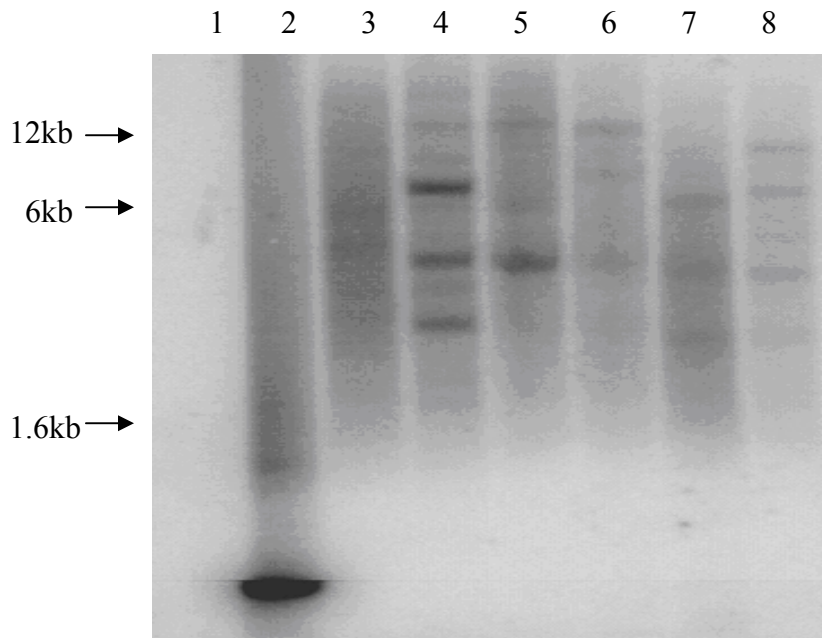


Figure 2.37. Southern blot analysis for chitinase genes in transgenic and non-transgenic Taipei 309 plants. Lane 1 was the negative control. Lane 2 was the positive control. Lane 3 was a non-transgenic control plant. Lanes 4, 5, and 8 were the transgenic plants 9-2, 46-1 and 33-3 from group 1. Lane 6 was the transgenic plant 33-4 from group 2. Lane 7 was the transgenic plant 15-7 from group 3.

2.3.3 Results of Greenhouse Tests with Transgenic and Control Plants

2.3.3.1 Hygromycin B Test Results

All tested transgenic plants with no lesion formed in response to exposure to hygromycin were in group 1. The rest of the tested plants were in three groups based on cluster analysis (Table 2.9). The resistant and non-resistant reactions could be readily distinguished. When compared with the non-transgenic control, transgenic plants in groups 1 and 2 had significantly shorter lesions. Transgenic plants in groups 3 and 4 had significantly longer lesions. Transgenic plants in groups 1 and 2 were not significantly different from the resistant control. Transgenic plants in groups 3 and 4 had significantly longer necrotic lesions. Plants in group 1 had resistance to hygromycin B equal to the resistant control plants. Some transgenic plants in group

2 appeared to be moderately resistant, but some non-transgenic plants were also in group 2 (Figure 2.38). This made it difficult to identify moderate resistance.

As in the field test, necrotic lesion length was significantly correlated with extended lesion length with a Pearson correlation coefficient of 0.8348 (P value < 0.0001). One hundred and thirty seven out of 165 transgenic plants tested in the greenhouse were highly resistance to hygromycin B.

2.3.3.2 Reaction of Transgenic and Non-transgenic Plants to Liberty Herbicide in Greenhouse Tests

One hundred and sixty one transgenic plants were tested with 363ppm Liberty herbicide in greenhouse tests. Sixty-six plants did not have lesions produced after exposure to Liberty and were placed into group 1. Ninety seven plants that produced lesions in response to exposure to Liberty were grouped into three groups based on cluster analysis (Figure 2.39). When compared with the non-transgenic susceptible control, transgenic plants in groups 1 and 2 had significantly shorter lesions and plants in group 4 had significant longer lesions (Table 2.10). When compared with the resistant control, only the plants in group 1 were not significantly different for lesion length as both groups produced no lesions. Transgenic plants in group 1 were highly resistant, but some plants in group 2 may have had a moderate level of resistance even though it was not clearly detected.

The length of necrotic lesions and extended lesions was significantly correlated with a Pearson correlation coefficient of 0.910 (P -value < 0.0001). The extended lesion was also analyzed. When compared with the non-transgenic control, transgenic plants in groups 1 and 2 had significantly shorter extended lesions, while transgenic plants in groups 3 and 4 had significantly longer extended lesions (Table 2.11).

Table 2.9. Multiple comparisons of susceptibility groups based on cluster analysis of mean lesion length, comparing resistant and non-transgenic susceptible control plants for reaction to hygromycin B in greenhouse tests.

Susceptibility group	Lesion length range (mm)	Number of plant	Mean lesion length (mm)	Standard error
1	0	137	0.0a ^x	0.0000
2	1- 6	19	2.5a	0.7602
3	9-19	5	14.8b ^y	1.4839
4	28 - 57	4	40.5b	1.6590
Resistant control		24	0.1	0.6773
Non-transgenic control		15	13.1	0.8567

^x significant at 0.05 level compared when compared with the non-transgenic control.

^y significant at 0.05 level compared when compared with the resistant control based on Dunnett's t Tests for lesion length.

Note: This test controls the Type I experimentwise error for comparisons of all groups against a control.

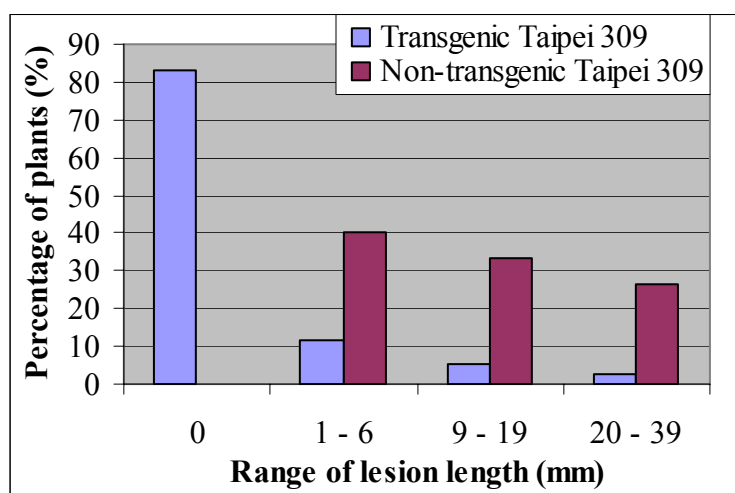


Figure 2.38. The percentage of plants in each susceptibility group based on cluster analysis of mean necrotic lesion length after exposure of transgenic and control plants to hygromycin B (200 ppm) in greenhouse tests.

Table 2.10. Multiple comparisons of susceptibility groups, based on cluster analysis of lesion length, when compared to resistant and non-transgenic susceptible control plants exposed to 363ppm Liberty herbicide in greenhouse tests.

Susceptibility group	Range of lesion length (mm)	Number of plants	Mean lesion length (mm)	Standard error
1	0	66	0.0a ^x	0.0000
2	4-38	22	27.7ab	2.1493
3	40-60	34	51.4b ^y	1.5744
4	62-93	41	71.1ab	1.7289
Non-transgenic control		17	56.1	2.4451
Resistant control		26	0.0	1.9771

^x significant at 0.05 level when compared with the non-transgenic control.

^y significant at 0.05 level when compared with the resistant control based on Dunnett's t Tests for lesions produced in response to Liberty herbicide.

Note: This test controls the Type I experimentwise error for comparisons of all groups against each control.

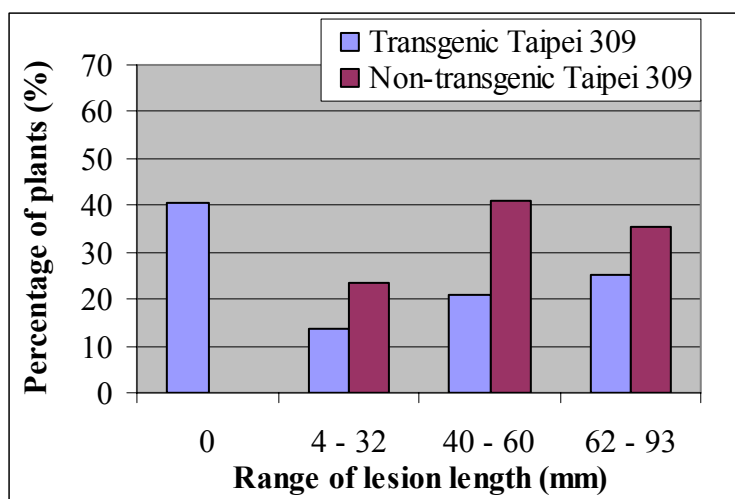


Figure 2.39. The percentage of plants in each susceptibility group based on lesion length after exposure to 363ppm Liberty herbicide in greenhouse tests.

When compared the with resistant control plants, transgenic plants in group 1 did not have significantly longer extended lesions as they had no lesions, but transgenic plants in groups 2, 3, and 4 had significant longer extended lesions. Fifty five transgenic plants with no extended lesions also had no necrotic lesions. These 55 plants with no necrotic lesions or extended lesions had resistance to Liberty herbicide equal to the resistant control. Eleven transgenic plants with no necrotic lesions and limited extended lesions (4mm, 5mm, 5mm, 5mm, 10mm, 21mm, 25mm, 35mm, 35mm, 46mm, and 47mm) and three resistant control plants with no necrotic lesions and limited extended lesions (5mm, 15mm, and 22mm) may have been a little less resistance, but it could not be detected by statistical analysis. All non-transgenic plants had extended lesions and were in placed into groups 2, 3 and 4 (Figure 2.40).

One hundred and fifty seven transgenic plants were also tested with Liberty herbicide at 750ppm in the greenhouse tests. There were no differences in lesion length or extended lesion length after exposure to 750ppm and 360ppm Liberty herbicide (Table 2.12).

2.3.3.3 Sheath Blight Test Results

The average heights of transgenic and non-transgenic plants were 306mm and 359mm, respectively. The t value was 2.58 with P-value 0.011, which was significantly different at a marginal level. The correlation of lesion length with ratio of lesion length to plant height was significantly correlated with a Pearson correlation coefficient of 0.927 (P-value < 0.0001).

Eighty-eight transgenic plants were successfully inoculated in the greenhouse tests. Ten plants with a ratio of lesion length to plant height less than 1 were grouped together, and the other 78 plants were divided into three groups by cluster analysis.

Table 2.11. Multiple comparisons of susceptibility group based on cluster analysis of extended lesion length when compared to resistant and non-transgenic control plants exposed to 363ppm Liberty herbicide in greenhouse tests.

Susceptibility group	Range of extended lesion (mm)	Number of plants	Mean extended lesion length (mm)	Standard error
1	0	55	0.0a ^x	0.0000
2	4-67	26	35.7ab ^y	3.1613
3	73-122	63	98.8ab	2.0309
4	147-245	19	153.8ab	3.6980
Non-transgenic control		17	83.3	3.9092
Resistant control		26	5.8	3.1613

^x significant at 0.05 level when compared with the non-transgenic control.

^y significant at 0.05 level when compared with the resistant control based on Dunnett's t Tests for extended lesion length after exposure to Liberty herbicide.

Note: This test controls the Type I experimentwise error for comparisons of all groups against each control.

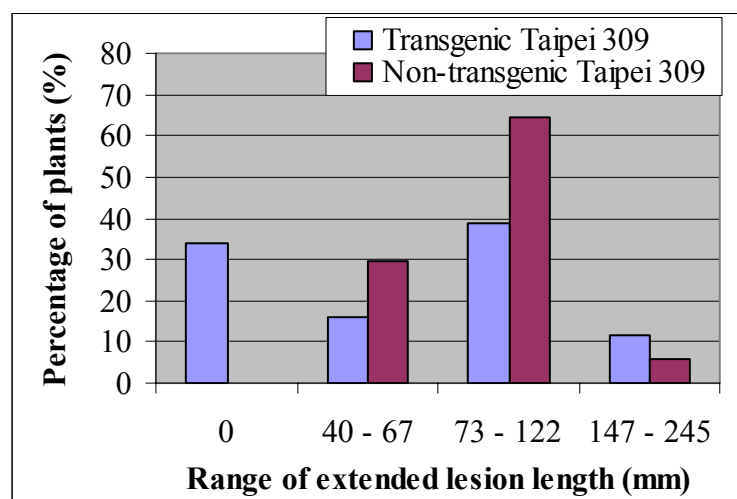


Figure 2.40. The percentage of plants in each susceptibility group based on cluster analysis of transgenic and non-transgenic TP309 plant's extended lesion length after exposure to 363ppm Liberty herbicide in greenhouse tests.

Table 2.12. Paired t test for mean lesion length and mean extended lesion length when transgenic plants were exposed to 750ppm or 363ppm Liberty herbicide.

	Mean difference when tested with 750ppm or 363ppm Liberty herbicide	t	P value
Necrotic lesion	2.07	1.8261	0.0697
Extended lesion	3.23	1.3670	0.0928

When compared with susceptible non-transgenic control plants, transgenic TP309 plants in groups 1 and 2 had significantly less disease, and group 4 plants had significantly more disease. Plants in group 3 were not significantly different (Table 2.13). There was a similar result when transgenic plants were compared with susceptible Cocodrie control plants. Transgenic Taipei 309 plants in groups 1 and 2 had significantly less disease, and group 4 plants had significantly more disease. More importantly, susceptible non-transgenic Taipei 309 plants were not significantly different from the susceptible Cocodrie control plants, which was not the case in the field test. No non-transgenic Taipei 309 and Cocodrie plants were placed into group 1. Plants in group 1 had higher levels of sheath blight resistance (Figure 2.41). The lowest ratio value of non-transgenic plants was 1.54. There were nine transgenic plants in group 2 that had ratio values less than 1.54, so these plants may have some level of resistance.

Based on cluster analysis, 18 transgenic plants with less than or equal to 4mm mean lesion length were grouped together, and the other 70 plants were grouped into three groups (Figure 2.42). When compared with susceptible non-transgenic plants, transgenic plants in groups 1 and 2 had significantly shorter lesion lengths. Plants in group 3 were not significantly different. Plants in group 4 had significantly longer lesion lengths. The results were similar when transgenic plants were compared with the susceptible Cocodrie control (Table 2.14). There was not a significant difference between non-transgenic Taipei 309 and Cocodrie plants.

Table 2.13. Multiple comparisons of susceptibility groups based on cluster analysis of the ratio of lesion length to plant height when compared to non-transgenic control plants in a greenhouse sheath blight test.

Susceptibility group	Ratio range	Number of plants	Mean ratio	Standard error
1	0-1	10	0.7a ^x b ^y	0.8030
2	1.06-4.06	46	2.4ab	0.3744
3	4.6-11.92	28	7.1	0.4799
4	19.46-30.36	4	23.9ab	1.2697
Non-transgenic control		22	6.1	0.5414
Cocodrie		20	5.9	0.5678

^x significant at the 0.05 level when compared with non-transgenic Taipei 309.

^y significant at the 0.05 level when compared with the susceptible Cocodrie plants based on Dunnett's t Tests for lesion length.

NOTE: This test controls the Type I experimentwise error for comparisons of all groups against a control.

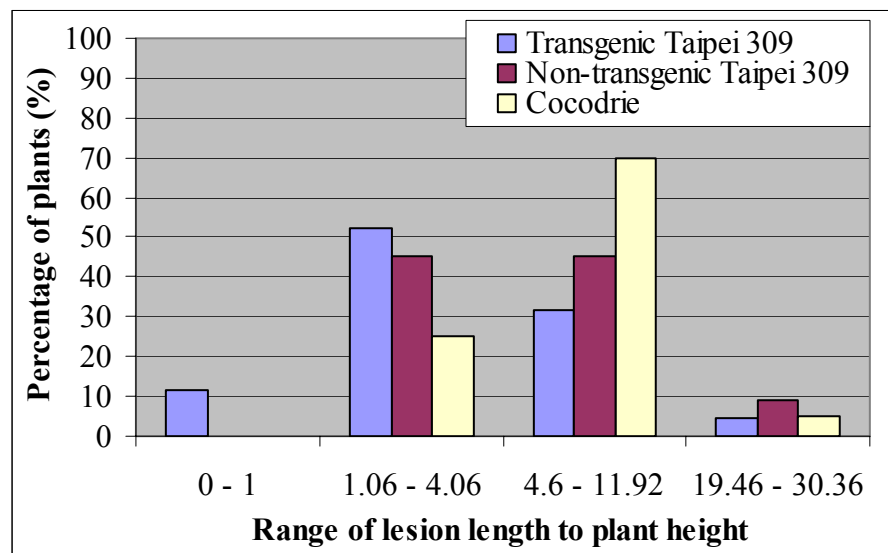


Figure 2.41. The percentage of plants in each susceptibility group based on cluster analysis of ratio of lesion length to plant height for a sheath blight test in the greenhouse.

Table 2.14. Multiple comparisons of susceptibility groups based on cluster analysis of lesion lengths with transgenic plants compared to non-transgenic susceptible control plants in a sheath blight test in the greenhouse.

Susceptibility group	Range of lesion lengths (mm)	Number of plants	Means of lesion lengths (mm)	Standard error
1	0-4	18	2.9a ^x b	2.3416
2	5-16	51	9.5ab ^y	1.3911
3	18-38	16	26.1	2.4837
4	70-85	3	75.3ab	5.7358
Non-transgenic plants		22	23.5	2.1181
Cocodrie control		20	26.7	2.2215

^x significant at the 0.05 level when compared with non-transgenic Taipei 309.

^y significant at 0.05 level when compared with the susceptible Cocodrie control. based on Dunnett's t Tests for lesion length.

NOTE: This test controls the Type I experimentwise error for comparisons of all groups against a control.

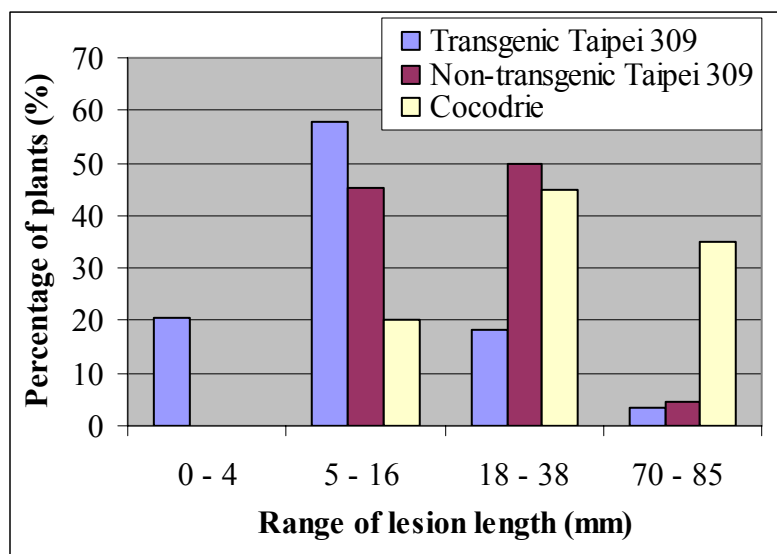


Figure 2.42. The percentage of plants in each susceptibility group based on cluster analysis of lesion lengths in a sheath blight test in the greenhouse.

Ten transgenic plants placed in group 1 based on cluster analysis of ratio values (94-1, 111-3, 89-3, 74-1, 88-4, 86-3, 98-1, 59-1, 54-1, and 86-2) were also placed in group 1 based on cluster analysis of lesion length. The remaining eight transgenic plants in group 1 based on lesion length (89-4, 77-2, 95-4, 79-1, 82-4, 95-3, 82-3, and 50-1) also included transgenic plants with ratio values less than the least ratio of 1.54 for non-transgenic plants. These plants had been placed in group 2 based on analyses of ratio values and had lower levels of resistance. Only one transgenic plant (81-2) had a ratio value less than 1.54, but it was placed in group 2 based on lesion length. Therefore, both methods were very similar for detecting high levels of sheath blight resistance in greenhouse tests. Groups based on lesion length tended to have more plants than groups based on ratio values. Both methods could be used to distinguish resistant plants.

When the susceptible Cocodrie plants were grouped, plants in groups 1, 2, 3, and 4 were 0%, 25%, 70% and 5%, respectively, when grouping by ratio values. When grouping by lesion length, the groups had 0%, 20%, 45% and 35%, respectively. It is clear that more Cocodrie plants were placed in group four when analyzing lesion length by cluster analysis. There was not a significant difference between Cocodrie and non-transgenic Taipei 309 plants in lesion length.

2.4 DISCUSSION

Sheath blight is considered the second most important disease on rice worldwide after the rice blast disease (Lee and Rush, 1983). Many research avenues have been explored to control this disease. Some pesticides are available, but they are expensive and not available in many developing countries. To date, only partial resistance to SB has been found worldwide. Using transformation techniques to transfer PR genes for fungal disease resistance has been used in many plant species and may have application for controlling SB on rice.

Co-transformation has been successfully used in biotechnology (Chen et al. 1998, Lyznik et al. 1989, Tang et al. 1999). Transgenic plants with the *bar* gene for resistance to Liberty herbicide or hygromycin B and sheath blight resistance genes would be very useful for breeders selecting disease resistance using products of the *hpt* or *bar* genes as markers.

2.4.1 Field Test

In these tests plant heights of transgenic Taipei 309 plants did not differ significantly from the height of non-transformed Taipei 309 control plants. This suggests that clonal variation did not occur among these transgenic plants as height variation is one of the most common trait changes in cultured rice. Changes observed for resistance to hygromycin B and Liberty herbicide and for resistance to *R. solani*, cause of the rice SB disease is unlikely to be due to clonal variation during the plant regeneration through tissue culture process.

2.4.1.1 Hygromycin B Test

As hygromycin B was used in all the steps of the callus selection and plant regeneration process during transformation, only resistant callus survived during the selection process. All plasmids used in these studies had the *hpt* gene. For these reasons, most of the regenerated plants showed resistance to hygromycin B in the field testing of transgenic plants. The phenotypic traits lesion length and extended lesion length showed significant correlation in the field test. One of these methods can be chosen for future field testing of putatively transformed plants. The measurement of necrotic lesion length is sufficient.

2.4.1.2 Liberty Test

The five most strongly resistant plants (41-1, 41-2, 44-1, 46-1 and 21-2) had the same reaction to both 363 and 750ppm Liberty ai in the field tests. Other transformed plants had significantly different reactions to these two Liberty concentrations. The same resistant plants

were also highly resistant to hygromycin B. A PCR test showed that all of these plants were transformed with the *bar* and *hpt* genes. Other plants expressed the Liberty resistance genes, but not the hygromycin resistance gene. At least one plant did not express the *hpt* gene even though both genes were in the same plasmid DNA used for transformation.

2.4.1.3 Sheath Blight Test

The grouping methods based on cluster analysis of lesion length/plant height ratios and lesion length for determining sheath blight resistance gave similar results, especially for the resistant group 1. This is very important for identifying truly resistant plants in future transformation studies. Plants of the susceptible variety Cocodrie had severe sheath blight after inoculations in the field, which indicated that both the inoculation method and the environment were suitable for disease development when this test was conducted.

The lesion length/plant height ratios and measured lesion lengths were highly correlated with the 0-9 disease rating on the same plants at maturity. This suggested that disease development on individual transformed plants was consistent throughout the season. Plants that had resistant disease measurements early in the season also gave a resistant reaction late in the season confirming that they were really resistant.

The lesion length data from the first and second inoculations was not statistically correlated indicating that disease development was not consistent for transgenic plants inoculated at different times during the season. Expression of the transgene may be different at different stages of growth. The second inoculation was near the end of the season and the environmental conditions may not have been as favorable for disease development as earlier in the season. Inoculation early in the season to determine resistance is more effective.

Both the hygromycin B and Liberty resistance tests gave clear reactions for separating resistant or susceptible plants. Plants transformed with the beta glucanase and chitinase genes also had either or both of the *hpt* or *bar* genes expressing. The presence of these genes in plants that are SB resistant from transformation, would allow for easy selection of SB resistant plants from segregating populations if all the genes have been inserted at the same location in a chromosome (Chen et al., 1998). After crossing the transformed plant with a non-transformed plant of a SB susceptible variety, the F₂ population could be sprayed with Liberty herbicide or seedlings exposed to hygromycin B and surviving plants would also be SB resistant. The five most resistant R₀ plants from these studies were crossed with the SB susceptible variety Cocodrie. These plants were also resistant to Liberty herbicide and hygromycin B. Segregating F₂ populations from these crosses will be tested for SB, Liberty, and hygromycin resistance in the 2004 season by the rice pathology group to see if any of these co-transformed plants had the PR and selection genes inserted in the same location in the same chromosome (linked). If such a plant can be located among the transgenic plants generated in these studies, this would be an extremely important contribution.

In this study, 17 transgenic Taipei 309 plants were in the SB resistant group 1 based on SB lesion length. Fourteen out of these 17 plants were also hygromycin B resistant and 10 of these plants were resistant to Liberty herbicide.

PCR and southern analysis for both β -1, 3-glucanase and chitinase indicated that these genes were present in the tested transgenic plants which had different levels of resistance. Plants with high level resistance, but without the transgenes have not been found, which indicated the resistance was from the transgenes. Plants with the transgenes but not showing high level resistance indicated gene silencing.

2.4.2 Greenhouse Test

2.4.2.1 Hygromycin B Test

One hundred and thirty seven out of 165 tested transgenic plants had strong resistance to hygromycin B with no necrotic lesions and limited extended lesion development. One transgenic plant (56-6) with no necrotic lesion and a 6.35mm extended lesion may have a slightly lower level of hygromycin B resistance. Among 24 resistant control plants, there was one resistant control plant (C4-3-7-2) with no necrotic lesion and a 2mm extended lesion, one resistant plant (C4-3-5-2) with a 1mm necrotic lesion and a 1mm extended lesion, and one resistant plant (C4-3-4-2) had a 1mm necrotic lesion and a 6mm extended lesion. Thus, resistance levels varied slightly among the resistant control plants, but transgenic plants with resistance similar to the resistant control plants were considered resistant.

2.4.2.2 Liberty Test

Fifty one out of 55 strongly Liberty resistant plants were also hygromycin B resistant. Therefore, most Liberty resistant plants were also hygromycin B resistant, which was consistent with the field test results.

The differences in development of necrotic lesions after exposure to Liberty concentrations of 750 and 363ppm, and the differences in extended lesions for liberty concentrations of 750 and 363ppm were not significantly different. This may be because the environmental conditions in the greenhouse were less severe than in the field. Molecular testing is very important to prove that the resistance, especially moderate resistance, was from the transgene.

2.4.2.3 Sheath Blight Test

Ten transgenic plants placed in group1 based on ratio values were resistant to sheath blight, 9 out of the 10 plants were hygromycin B resistant, and 7 of the 10 plants were also Liberty resistant.

Using the ratio of lesion length to plant height, or lesion length measurements, transgenic plants in group 1 had significantly less SB disease than non-transgenic Taipei 309 and Cocodrie control plants, but non-transgenic Taipei 309 plants had significantly less SB disease compared to the susceptible Cocodrie control. This indicated that environmental factors in the greenhouse were less favorable for disease development compared to field conditions, even though a humidity chamber was used. Thus, data developed in greenhouse tests should be carefully evaluated.

CHAPTER 3

CO-TRANSFORMATION OF THE THIONIN AND *bar* GENES TO LAFITTE RICE FOR OBTAINING BACTERIAL PANICLE BLIGHT AND LIBERTY HERBICIDE RESISTANCE

3.1 INTRODUCTION

Thionins are proteins found in different tissues of many plant species that have toxic to microorganism and antimicrobial properties (Melo et al., 2002). Leaf thionin of barley has been reported as lower molecular weight polypeptides that may play an important role in defending barley against plant pathogens (Andresen et al. 1992). It can be isolated from cell walls and vacuoles of barley leaves (Hohlmann et al., 1988). The leaf-specific thionins of barley are encoded by a complex multigene family on chromosome 6, and they are toxic to plant pathogenic fungi (Bohlmann et al., 1988).

Holtorf et al. (1998) showed that transgenic *Arabidopsis thaliana* plants that expressed thionin were more resistant to infection by *Plasmodiophora brassicae*. Rice transformed with the gene for oat thionin, and accumulating high levels of thionin in cell walls, were more resistant to seedling disease caused by *Burkholderia plantarii* and grew almost normally, while wild-type rice seedlings were wilted and severely blighted (Iwai et al., 2002).

Bacteria panicle blight has become an important disease in Louisiana, and there has not been an effective pesticide available to control this disease (Rush, 1998; Shahjahan et al., 2000). Developing disease resistance in varieties using transformation is becoming a major research tool for plant disease control. Further, as was reported by Chen et al. (1998), co-transformation is a useful way to transfer multiple genes to rice, and it may be possible to obtain disease and herbicide resistance in the same plants.

3.2 MATERIALS AND METHODS

3.2.1 Plasmids for Transformation

The plasmid pMTHY has the barley leaf thionin gene (approximate 1000bp from Dr. Ding Shih's laboratory in the Biochemistry section of the Department of Biological Sciences at Louisiana State University) with the 35S cauliflower mosaic virus promoter and the *hpt* gene with the 35S promoter (Gatz and Quail, 1988). The plasmid pUBIBarHy has the *bar* gene (615bp) (Wohlleben et al., 1988) for resistance to Liberty herbicide, which was cloned from *Streptomyces viridochromo*, with the maize ubiquitin promoter (Christiane and Quail, 1988) and the *hpt* gene (Zalacain et al., 1986) also with the maize ubiquitin promoter. The plasmids were transformed to competent cells of *Escherichia coli*. These plasmids were provided by Dr. Ding S. Shih's laboratory in the Department of Biological Sciences at Louisiana State University.

3.2.2 Transformation Device and Tissue Culture Media

In January of 2000, Dr. X.Y. Sha co-transferred the plasmid with the *bar* gene and the plasmid pMTHY, with the thionin gene, at the molar ratio 1:1 to calli derived from Lafitte rice using particle bombardment. A Biolistic® PDS-1000/He device from BioRadTM was used for transferring the plasmid DNA to rice calli. The transformation procedures used were the same as those described in Chapter 2 of this Dissertation.

Six different media were used. A callus induction medium (CI) was used to induce calli from the scutella of mature rice seeds. An osmotic medium was used to create high osmotic pressure in each cell of the calli so they would take up plasmid DNA efficiently. A selection medium containing the antibiotic hygromycin B was used to select transformed cells which expressed the *hpt* gene. Other media included a pre-regeneration medium (PR) used to mature embryogenic calli and a regeneration medium used to regenerate plants from transformed calli,

and a rooting medium was usually required to allow plantlets produced on calli to develop roots to the point that they could be transferred to soil in the greenhouse.

The components of each medium were the same as described in Chapter 2 section 2.2.1 of this Dissertation.

3.2.3 Regeneration of Transgenic Plants

Calli were generated from the scutellum of mature seeds that were first dehulled, surface sterilized in 1.5% sodium hypochloride solution for 30 minutes, then plated on callus generation medium and incubated in the dark at 27°C. Small calli were transformed as outlined in section 2.25, Chapter 2 of this Dissertation.

Transformed calli were transferred to PR medium after the selection of calli on hygromycin B selection medium. The calli were incubated on PR medium for 10-14 days at 27°C with a 12hr light and 12hr dark regime. Then selected calli were transferred to regeneration medium and subcultured on the regeneration medium every 2 weeks using the same culture conditions. Green spots appeared on calli after about 20 days on regeneration medium. Some of the green spots produced shoots and developed into plantlets. The plantlets were then transferred to the rooting medium. After root systems were established, the plants were transplanted to a soil mix (1 soil : 2 sand : 1 peat moss) in 8 inch plastic pots in a greenhouse.

3.2.4 Greenhouse and Field Screening for Resistance to Hygromycin B, Liberty Herbicide, and *Burkholderia glumae*

The hygromycin B and Liberty herbicide screening procedures were the same as described in sections 2.2.6 and 2.2.7 of Chapter 2 of this Dissertation. One leaf each from transgenic and non-transgenic control plants were tested with hygromycin B at 200ppm ai and Liberty herbicide at 750ppm ai. Plants with treated leaf blades with no necrotic or extended lesions were considered to be resistant.

Five R₀ plants were tested for resistance to hygromycin B, Liberty herbicide and *Xanthomonas oryza*, the cause of bacterial leaf blight in rice. The R₀ plants were harvested in December 18, 2000 from the greenhouse. After the panicles were dried in an oven at 42C for 2 days, the seeds were immediately planted on January 1, 2001 in the greenhouse to obtain seeds for future field plantings. Therefore, no tests were conducted on the R₁ plants. The R₁ plants were harvested to provide R₂ seeds on April 19, 2001 from the greenhouse and harvested panicles were again dried in a 42C oven for 2 days.

R₂ seeds were germinated in Petri dishes on April 30, 2001. On May 15, 2001 more R₂ seeds were germinated and non-transgenic Lafitte seeds were also germinated in Petri dishes. R₂ seedlings were transferred to soil in pots in the greenhouse on May 15, 2001 and May 28, 2001. All 52 R₂ plants and 12 non-transgenic Lafitte plants were transplanted to the field at the Louisiana State University Rice Research Station in Crowley, LA on June 14, 2001. The R₂ plants were space-planted in groups of progeny from the original five R₀ plants. The R₂ plants and non-transgenic control plants were inoculated with *B. glumae*, the causal agent of bacterial panicle blight, by spraying the panicles as they were half emerged from the panicles with a suspension (at concentration about ca. 10⁸ CFU) of bacterial isolate # 951886-4-1c. A disease rating was given to each plant at maturity, and the inoculated panicles were harvested (R₃ seeds).

R₃ seeds were planted in a greenhouse on November 9, 2001 and R₃ plants were tested for resistance to hygromycin B and Liberty herbicide as described in sections 2.27 and 2.26, Chapter 2 of this Dissertation. The cut leaf method (described in section 3.2.4.1 below) was used to test with *Xanthomonas oryzae*, and the injection method (described in section 3.2.4.2 below) was used in the greenhouse to test seedlings with *B. glumae*. R₃ plants were harvested (R₄ seeds) on April 1, 2002 and May 16, 2002.

R₄ seeds were planted in a greenhouse on April 14, 2003, and the resulting plants R₄ were transplanted to the field at the LSU Rice Research Station at Crowley, LA on May 21, 2003. R₄ plants in the field were tested for resistance to hygromycin B and Liberty herbicide as described in sections 2.27 and 2.26, Chapter 2 of this Dissertation. Spraying *B. glumae* on half emerged panicles was used to test the resistance to panicle blight (described in section 3.2.4.2 below).

3.2.4.1 Bacterial Leaf Blight Test

Xanthomonas oryza, causal agent of bacterial leaf blight (BLB), was reported from Louisiana (Rush et al., 1988; Jones et al., 1989). This pathogen has the potential for causing serious yield loss in Louisiana rice and was included along with *Burkholderia* spp. in resistance testing of Lafitte rice plants transformed with the gene for thionin production. The medium for culturing bacterial isolate *Xanthomonas oryza*-17 (provided by Dr. Chris Clark, Department of Plant Pathology and Crop Physiology, Louisiana State University at Baton Rouge, LA.) was Wilbrinck's medium [Agar 20.0g, Sucrose 10.0g, Peptone 5.0g, K₂HPO₄ 0.5g, MgSO₄·7H₂O 0.25g, Na₂SO₃ 0.05g in 1 liter deionized water maintained at pH 7.2] (Atlas, 1993). Bacteria were streaked onto solid medium and cultured overnight at 28C. Bacteria from each plate were washed into 200ml of sterile water for greenhouse testing. The cut leaf-tip method (cut each leaf tip while immersed in bacterial culture solution with ca. 10⁸ CFU/ml, and leave for 3-5 seconds) was used to test for resistance to BLB. One leaf on each transgenic and non-transgenic control plant was tested. There were 5 R₀ plants and 92 R₃ transgenic plants. As BLB has not caused a major disease problem in Louisiana, the test was only conducted in the greenhouse.

3.2.4.2 Seedling and Panicle Inoculations with *B. glumae* on Transgenic and Non-transgenic Control Plants

B. glumae was cultured on solid King's B medium [Agar 20g, Proteose peptone 20g, K₂HPO₄ 1.5g, MgSO₄·7H₂O 1.5g, Glycerol 15mL at pH 7.2] (Atlas, 1993) overnight at 28C.

Bacteria on each plate were then washed into 200ml sterile deionized water.

R₂ seeds were planted in the greenhouse, and all the established plants were transplanted to the field in summer 2001. There were a total of 48 transgenic plants in 3 rows from 12 parents and 12 non-transgenic plants in one row in the field. The row spacing was 25.4cm and plant spacing was 10cm. NPK (24-13-13) fertilizer was applied at 500 lb/acre just after transplanting. When panicles were in the half-emerged stage, *B. glumae* was sprayed as in the nursery. The disease on each plant was evaluated in the field for BPB disease levels at maturity as severe or moderate (personal communication with Dr. M.C. Rush and Dr. A.K.M. Shahjahan).

In a November 2001 greenhouse test, R₃ seeds were planted in 203mm diameter plastic pots with a ratio of 1 soil : 2 sand : 1 peat moss. Ninety three transgenic R₃ and 18 non-transgenic seedlings were injected with *the B. glumae* (at a concentration of ca.10⁸ CFU/mL) about 2.54cm from the top of each plant on the sheath to test for resistance. Necrotic lesion formation was evaluated at 8 days after inoculation. The lesion lengths were not measured, as the lesions were irregular.

In the 2003 season, a field test was conducted to test R₄ generation transformed Lafitte plants for resistance to BPB. The seeds were planted in pots the greenhouse and then transplanted to the field on May 21, 2003. There were 24 rows of transgenic Lafitte plants, 3 rows of non-transgenic plants and 4 rows of transgenic Taipei 309 plants used as the hygromycin and Liberty resistant controls. There were 10 plants transplanted into each row initially, but some of the plants died after transplanting.

Hygromycin and Liberty resistance screening tests procedures were the same as described in sections 2.2.6 and 2.2.7 of Chapter 2 of this Dissertation.

At approximately 50% panicle emergence, panicles were covered with a crossing bag (Figure 3.1) open at the top and stapled around the culm at the bottom and then inoculated with a 24hr culture of *B. glumae* (951886-4-1c) about ca. 10^8 CFU in sterilized water. The bacterial suspension was applied by spraying the bacterial suspension with a hand-atomizer/sprayer inside the bag. After inoculation each crossing bag was sealed with paper clips at the top (Figure 3.1). At maturity BPB was evaluated on each panicle based on the discoloration of each panicle and percentage of blighted florets (0%, 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80% and 90%) (Figure 3.2). After this evaluation the panicles were harvested and dried in an oven at 42C for 3 days. Panicle weight and the number of filled kernels on each panicle were determined. Data were analyzed using the SAS software package (SAS Institute, 2002).



Figure 3.1. Partly emerged panicles were covered with crossing bags in the field (left) and inoculated by spraying a suspension of *B. glumae* (10^8 CFU/ml) onto the panicles through the top of the open bag. The bags were then closed with paper clips. Picture on the right shows a closer view of the covered panicle immediately after inoculation.



Five % of florets blighted



Ten % of florets blighted



Thirty % of florets blighted



Forty % of florets blighted



Fifty % of florets blighted



Ninety % of florets blighted

Figure 3.2. Typical floret damage by bacterial panicle blight based on percentage of florets blighted.

3.2.4.3 Molecular Tests to Determine That the Thionin Gene Was Present in Transformed Plants

DNA extraction from leaf tissues from transformed Lafitte rice plants with the thionin, *bar*, and *hpt* genes was the same as described in Chapter 2 for leaf tissue from plants transformed with the beta glucanase and chitinase genes. Primers for *bar* gene: forward primer sequence was 5'-TACCATGAGCCCAGAACGA-3', reverse primer was 5'-TCAGATCTCGGTGACGGGC A-3', and size of the amplification product was 600bp. Primers for *hpt* gene: forward primer sequence was 5'-AGTTCGACAGCGTCTCCGA-3', reverse primer was 5'-TATTCCTTTGCC CTCGGACGA-3', and size of the amplification product was approximate 1kb. The forward primer sequence of thionin gene for PCR was 5'-TTCTCAAATGCCATCCTTC-3', the reverse

primer was 5'-CATGCACAAGAAGGCATGA-3', and size of the amplification product was 400bp. All primers were diluted to 10uM/uL.

The PCR thermocycler program for *bar* gene detection in the R₄ generation using PCR was the same as described in Chapter 2. The PCR thermocycler program for detecting the thionin gene in R₄ generation plants was as follows: one step at 94C for 5 minutes, 2 cycles with 94C for 1 minute, 54C (decreasing 1C/cycle) for 1 minute, 72C for 1 minute, 35 cycles with 94C for 1 minute, 52C for 1 minute, 72C for 1 minute, final step at 72C for 10 minutes and then hold the material at 4C.

RNA was extracted from rice leaves using a Qiagen® kit. One hundred mg of leaf tissue was ground in liquid nitrogen, the tissue powder was placed into a RNase-free, nitrogen-cooled 2uL micro centrifuge tube. Lysis buffer RLT (450uL) was added and the mixture was vortexed vigorously. The lysate was pipetted directly onto a QIA shredder spin column, placed into 2mL collection tubes, and centrifuged for 2 minutes at a maximum speed of 13,200rpm. The supernatant of the flow-through was transferred to a new microcentrifuge tube. Ethanol with half volume of the flow-through was added to the supernatant, and mixed immediately by pipetting. The supernatant mixture was applied to a RNeasy mini column, placed into a 2ml collection tube, centrifuged for 15 seconds at 13,200rpm, and the flow-through was discarded. Buffer RW1 (350uL) was pipetted into the RNeasy mini column, centrifuged for 15 seconds at 13,200rpm to wash, and the flow-through was discarded. Ten uL DNase I stock solution was added to 70uL buffer RDD, and mixed by gently inverting the tube. The DNase I 80uL mixture was added directly onto the RNeasy silica-gel membrane, and placed on the benchtop for 15 minutes at room temperature (25C). Buffer RW1 (350uL) was added to the RNeasy mini column and centrifuge for 15 seconds at 13,200rpm. The flow-through was discarded, and the RNeasy

column was transferred into a new 2ml collection tube. Buffer RPE (500uL) was pipetted onto the RNeasy column, centrifuged for 15 seconds at 13,200rpm to wash the column, and the flow-through was discarded. Another 500uL of RPE buffer was added to the RNeasy column, centrifuged for 2 minutes at 13,200rpm to dry the RNeasy silica-gel membrane. The RNeasy column was centrifuged again for 1 minute at 13,200rpm and the collection tube with the flow-through was discarded. The RNeasy column was transferred to a new 1.5mL collection tube, 30 to 50uL RNase-free water was directly pipetted onto the RNeasy silica-gel membrane, and the mixture was centrifuged for 1 minute at 13,200rpm to elute.

Reverse transcription (RT) was conducted using extracted RNA from different plant samples. Two uL oligo dT, 2uL 10xRT buffer, 4uL dNTP, 1uL RNase inhibitor, 1uL reverse transcriptase, 4uL RNase-free water, and 6uL extracted RNA sample were added together to produce RT product. The program for RT was 42C for 1 hour, 92C for 10 minutes, and hold at 4C.

Two uL RT product was mixed with 2.5uL buffer, 0.25uL dNTP, 3uL MgCl₂, 2uL primers, 14.75uL RNase-free water, and 0.5uL polymerase to do regular PCR for the thionin gene. The PCR Thermocycler program was: one step at 94C for 4 minutes, 32 cycles with 94C for 30 seconds, 54C for 30 seconds, 72C for 1 minute, final step at 72C for 3 minutes and hold at 4C.

3.3 RESULTS

3.3.1 Regeneration of Transgenic Plants

Lafitte calli were derived from scutellar tissues from dehulled, surface sterilized kernels (1.5% sodium hypochloride solution 30 minutes) plated onto solid callus induction medium. These calli were subcultured on solid callus induction medium. The calli to be transformed were

placed on osmotic medium over night after transfer from plates of callus induction medium. After transformation, the calli were selected on selection medium with 50mg/L of hygromycin, pre-regeneration medium, and the regeneration medium, five R₀ plants were regenerated. The five transgenic plants were from different transformation events. All five plants were transferred to the greenhouse for further tests.

3.3.2 Tests Conducted on R₀ Plants

3.3.2.1 Hygromycin B Resistance Test

When tested against hygromycin B, the five transgenic Lafitte R₀ plants did not have either necrotic or extended lesions (black arrow), which indicated strong resistance to hygromycin B. Non-transgenic Lafitte control plants showed dark brown lesions (purple arrow) or dried, necrotic lesions (blue arrow) (Figure 3.3).

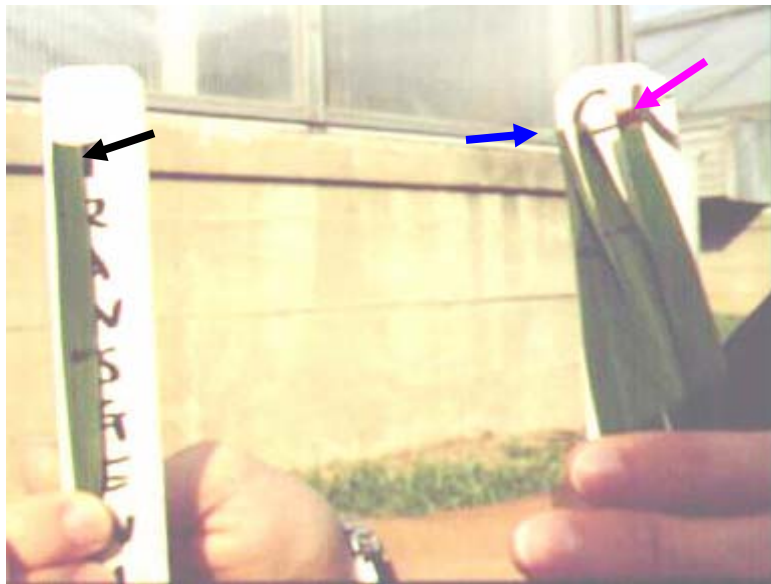


Figure 3.3. Transgenic Lafitte R₀ plants (left) and non-transgenic control plants (right) 4 days after exposure to hygromycin B.

3.3.2.2 Liberty Herbicide Resistance Test

Five days after exposure to Liberty herbicide, five transgenic Lafitte R₀ plants did not show necrotic or extended lesions. Non-transgenic Lafitte control plants had extended lesions up to 70mm long (Figure 3.4). Eleven days after exposure to Liberty herbicide, three transgenic plants (Lafitte 1, Lafitte 2, and Lafitte 5) had some discolored dots on the tips of tested leaves. Two plants (Lafitte 3 and Lafitte 4) did not show any symptoms, but tested leaves from non-transgenic plants had long necrotic lesions. Based on lack of development of necrotic lesions, the five transgenic plants had strong resistance to Liberty herbicide.

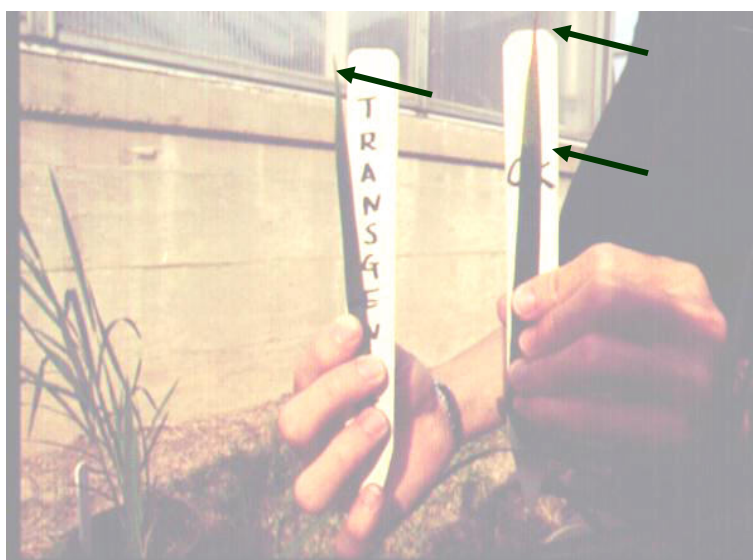


Figure 3.4. Symptoms expressed by transgenic Lafitte R₀ plants (left) and non-transgenic Lafitte plants (right) 4 days after exposure to Liberty herbicide.

3.3.2.3 Bacterial Leaf Blight Test

Eight days after inoculation with *X. oryza*, leaf-tip lesions on non-transgenic and transgenic Lafitte plants were not significantly different. The three non-transgenic Lafitte plants had lesions 1.2cm, 0.5cm and 0.4cm in length. The five transgenic Lafitte plants had lesions 0.2cm, 0.3cm, 0.2cm, 0.2cm, and 0.2cm in length. The mean lesion length for non-transgenic and

transgenic Lafitte plants were 0.7cm (standard error = 0.2517) and 0.26cm (standard error = 0.04), respectively. Statistical analysis showed there was no significant difference between the lesion means with t-value = 2.3 and p-value = 0.0612. Figure 3.5 shows the lesions 25 days after inoculation. Lesion lengths on transgenic (right) and non-transgenic control plants (left) still were not significantly different.

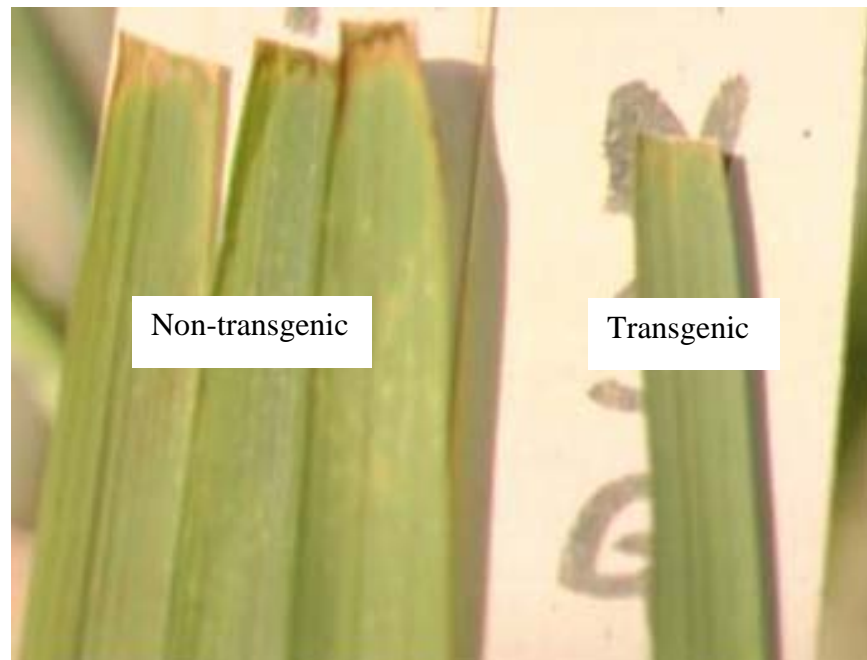


Figure 3.5. Transgenic R₀ plants and non-transgenic Lafitte plants 25 days after inoculating leaf tips with *Xanthomonas oryzae*.

3.3.2.4 Analysis for the *bar*, Thionin, and *hpt* Genes in Transformed and Non-transformed Lafitte Rice Plants Using PCR

PCR analysis of the five transgenic plants showed that all five plants were transformed with the *bar*, thionin and *hpt* genes (Figures 3.6, 3.7, and 3.8).

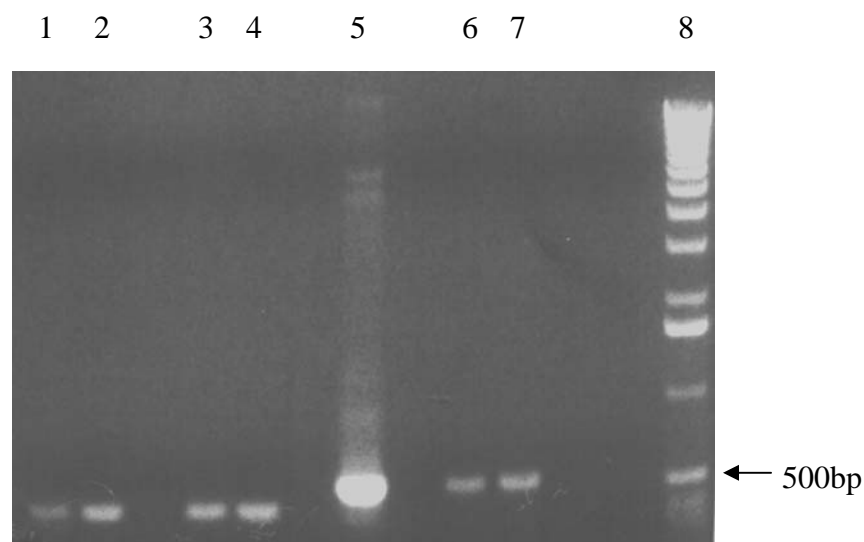


Figure 3.6. PCR analysis for *bar* and thionin genes from transgenic Lafitte rice. Lane 1: positive control for thionin gene; lanes 2 to 4: transgenic plants Lafitte 5, Lafitte 3 and Lafitte 2 for thionin gene; lane 5: positive control for bar gene; lanes 6 and 7: refer to transgenic plants Lafitte 4 and Lafitte 3 for *bar* gene; lane 8: 1kb marker.

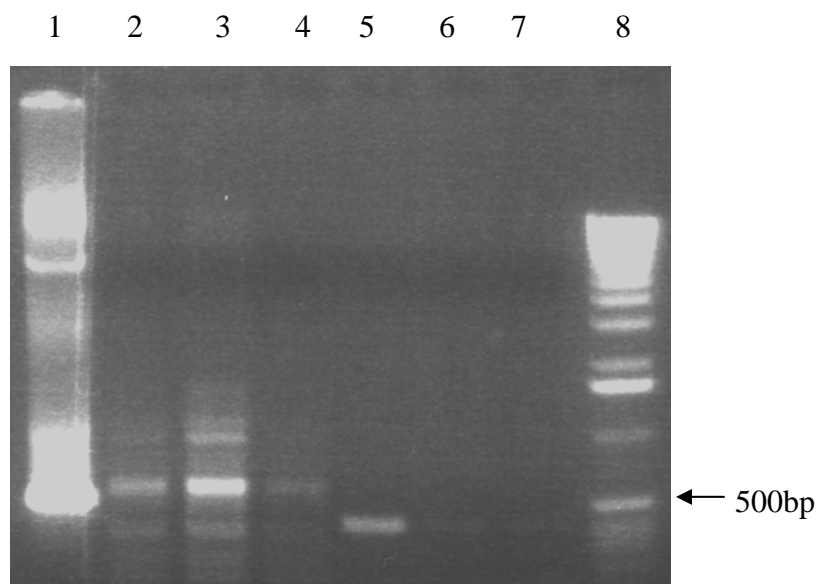


Figure 3.7. PCR analysis for *bar* and thionin genes from transgenic Lafitte plants. Lane 1: positive control for *bar* gene; lane 2 to 4: represent transgenic plants Lafitte 5, Lafitte 2 and Lafitte 1 for *bar* gene; lane 4: transgenic plant Lafitte 5 for thionin gene; lane 5: positive control for thionin gene; lanes 6 and 7: refer to transgenic plants Lafitte 4 and Lafitte 1 for thionin gene; lane 8: 1kb marker.

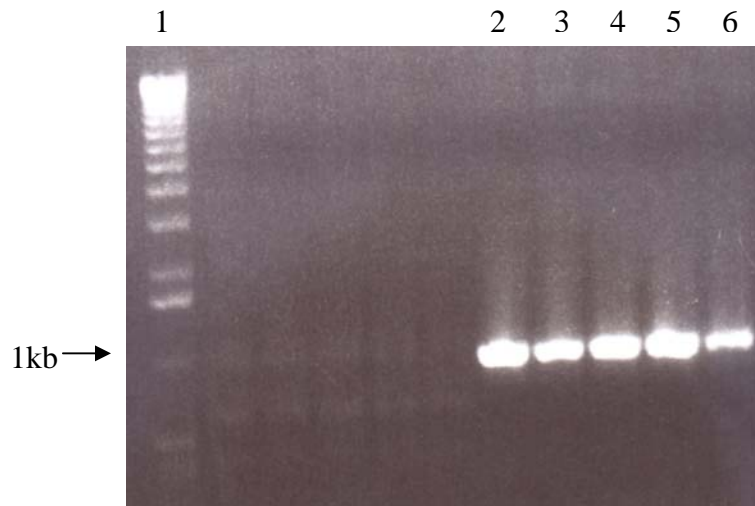


Figure 3.8. PCR analysis for *hpt* gene in transgenic Lafitte rice. Lane 1: 1kb marker; lanes 2 to 6: represent transgenic plants Lafitte 1, Lafitte 2, Lafitte 3, Lafitte 4 and Lafitte 5.

3.3.3 Field Testing of R₂ Plants

After spraying *B. glumae* on emerging panicles, the inoculated panicles were evaluated at maturity based on symptom development. All 12 non-transgenic Lafitte plants showed severe BPB symptoms. Thirty transgenic plants showed severe BPB symptoms and 22 transgenic plants had moderate panicle blight symptoms (Table 3.1). The ratio of resistant to susceptible plants was not 15:1 or 63:1, as found by Shao (2003). The segregation ratio, while not following inheritance rules, did not indicate that the thionin gene was not present in the susceptible transgenic plants. It is likely that gene silencing occurred in some of the transgenic plants.

Some transgenic R₂ plants with moderate resistance to BPB were used to extract RNA. RT-PCR was used to analyze the RNA and show expression of the thionin gene in some transgenic plants (Figure 3.9). Severely diseased transgenic plants will be tested at a later date.

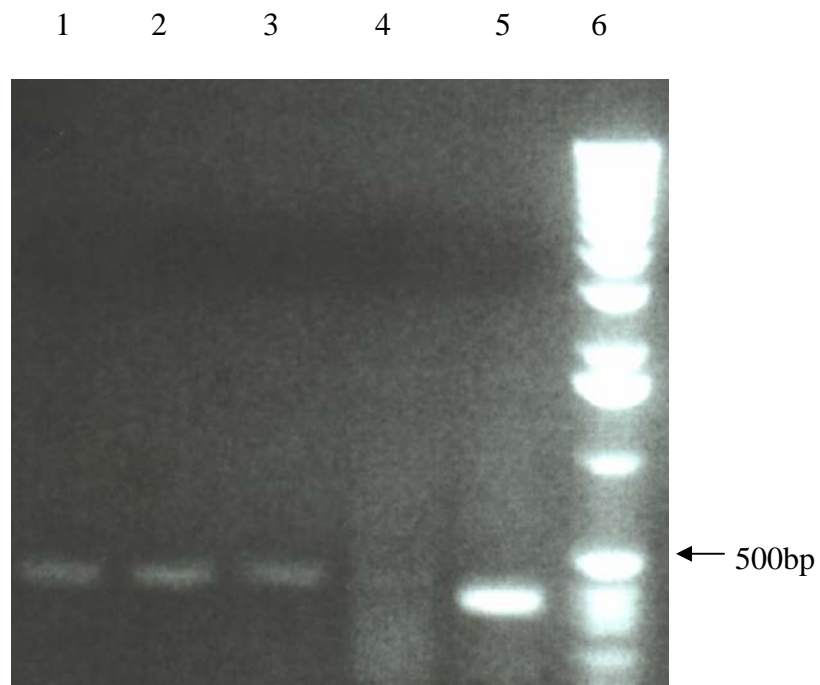


Figure 3.9. RT-PCR analysis for thionin gene in transgenic R₂ Lafitte plants. Lanes 1 to 3: represent 3 transgenic plants of Lafitte number 2-3; lane 4: a transgenic plant of Lafitte 2-6; lane 5: positive control; lane 6: 1kb marker.

Table 3.1. Transgenic plants with different levels of panicle blight symptoms corresponding to the RT-PCR results.

Plant	Disease levels	Number of plants	RT-PCR
Lafitte 1-1	Moderate (5)	1	
Lafitte 2-1	Severe	5	
	Moderate (3)	1	1 positive
Lafitte 2-2	Severe	3	
	Moderate	6	
Lafitte 2-3	Severe	2	
	Moderate (5)	10	3 positive
Lafitte 2-4	Severe	7	
Lafitte 2-6	Severe	4	
	Moderate (5)	1	1 positive
Lafitte 3-2	Severe	1	
	Moderate (5)	1	
Lafitte 3-4	Severe	8	
	Moderate (4, 5)	2	1 positive

3.3.4 Greenhouse Tests of R₃ Plants

3.3.4.1 Hygromycin B Test Results

Ninety two transgenic R₃ plants were screened for resistance to hygromycin B. Resistant plants had no necrotic or extended lesions while susceptible plants had long necrotic and/or extended lesions (Figure 3.10). Twenty transgenic plants were resistant to hygromycin B, and 8 transgenic R₃ plants were resistant to both hygromycin B and Liberty herbicide. The resistance did not segregate with typical ratios as described in Shao (2003). This was probably due to the *hpt* gene present in some susceptible plants being silenced.



Figure 3.10. Lesions on leaf blade tips of transgenic (right) and non-transgenic (left) Lafitte plants 7 days after treatment with hygromycin B in a greenhouse test.

3.3.4.2 Liberty Test Results

Ninety two R₃ transgenic plants were tested with Liberty herbicide. Resistant plants did not have necrotic or extended lesions, but susceptible plants, including non-transgenic plants,

had long necrotic and/or extended lesions (Figure 3.11). Ten transgenic Lafitte plants were resistant to Liberty herbicide and 8 more transgenic plants were resistant to both Liberty and hygromycin B. The Liberty resistance also did not follow normal segregation ratios.



Figure 3.11. Symptoms expressed 7 days after screening non-transgenic (left) and transgenic (right) Lafitte rice plants for resistance to Liberty herbicide in a greenhouse test.

3.3.4.3 BPB Screening Test Results

Ninety two R_3 transgenic plants were inoculated with *B. glumae* by injection. Plants with resistance to *B. glumae* showed small lesions around the injected area. Non-transgenic plants, susceptible to *B. glumae* had larger lesion (Figure 3.12). Fifty four transgenic plants showed different levels of resistance to *B. glumae*. Plants with resistance to both Liberty and *B. glumae* were not found. But this did not mean that both genes were not in the transgenic plants. Gene silencing may have occurred and the greenhouse seedling test may be less sensitive than the field panicle inoculation tests. Thus, further field tests on individual panicles were necessary.

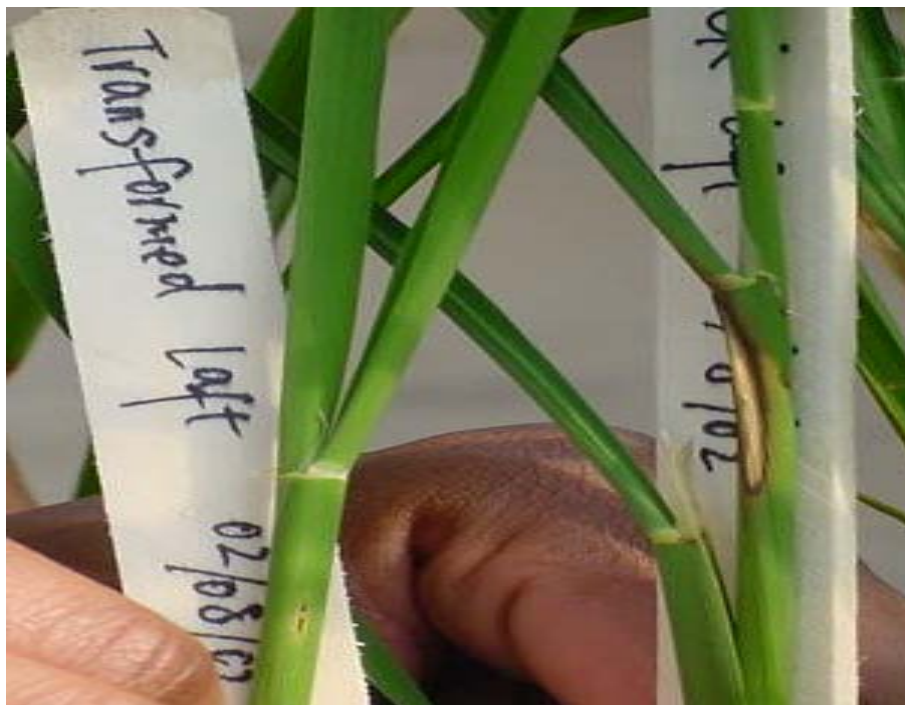


Figure 3.12. Seedling symptoms on transgenic R₃ and non-transgenic Lafitte plants 8 days after inoculation with *B. glumae*.

3.3.4.4 BLB Test Results

The BPB symptoms on R₃ transgenic and non-transgenic Lafitte plants were not significantly different, a result similar to that of the R₀ plants. Figure 3.13 illustrates the difference between the lesions on transgenic and non-transgenic Lafitte plants. This result further supported our conclusion that Lafitte may have natural resistance to BLB.

3.3.5 Field Tests on Transgenic R₄ Plants

Seeds from transgenic R₃ plants with resistance to *B. glumae*, hygromycin B, and Liberty herbicide were planted in the greenhouse, and then transplanted to the field at the LSU Rice Research Station, in Crowley, LA for testing of transgenic R₄ Lafitte plants for various resistances in a field test.



Figure 3.13. Necrotic lesions 25 days after leaf-tips were inoculated with *Xanthomonas oryzae* on transgenic (right) and non-transgenic R₃ Lafitte rice plants (left) in a greenhouse test.

3.3.5.1 Hygromycin B Tests

The progeny of transgenic plants resistant to hygromycin B were also resistant to hygromycin B (Figure 3.14). Transgenic plants in the same row had the same reaction in the hygromycin B test, either resistant or susceptible. This indicated that the progeny from individual transgenic plants of the R₃ generation were not segregating in the R₄ generation for the *hpt* gene. Fifty nine progeny rows with a total of total 511 transgenic plants were exposed to hygromycin B. Twelve progeny rows with 125 transgenic plants were resistant and 47 progeny rows with 386 transgenic plants were not resistant. Among the 12 progeny rows with resistant plants, five rows (42 plants) were from hygromycin B resistant plants in the previous generation (R₃), four rows were from hygromycin B and Liberty resistant plants in the previous generation (R₃), and three rows were from plants resistant to Liberty herbicide in the R₃ generation.



Figure 3.14. Transgenic plants with resistance to hygromycin B.

The three progeny rows with plants resistance to Liberty were not resistant to hygromycin B in the R_3 generation, but were resistant to hygromycin B in the R_4 generation. This suggested that gene expression was complicated or that previously silenced genes began expressing in the next generation.

Seven progeny rows (69 plants) from hygromycin B resistant plants in the previous generation were not resistant in the R_4 generation. One progeny row (8 plants) from a transgenic plant with resistance to both hygromycin B and Liberty in the R_3 generation did not have resistance to hygromycin B in the R_4 generation. This may be because the greenhouse conditions were not as favorable for symptom expression as the field. Plants with apparent resistance in the greenhouse may not show resistance in the field. Gene expression under different environmental conditions may not be the same and as a result, the resistance levels may vary.

3.3.5.2 Liberty Herbicide Tests on R₄ Generation Plants

As with the hygromycin test, transgenic plants in the same progeny row had the same reaction to Liberty herbicide, either resistant or susceptible (Figures 3.15 and 3.16). The *bar* gene present in individual plants in the R₃ may not be present in the R₄ generation. Three progeny rows from plants with Liberty herbicide resistant in the R₃ generation were also resistant in the R₄ generation. Four progeny rows from plants resistance to both Liberty and hygromycin B in the R₃ generation were also resistant in the R₄ generation. But one progeny row from a plant with resistance to Liberty herbicide in the R₃ generation and one progeny row from a plant with resistance to both hygromycin B and Liberty herbicide in the R₃ generation were not resistant to Liberty herbicide in the R₄ generation. This may indicate that resistance in greenhouse tests may differ from field tests because of environmental conditions during testing, or gene silencing may have caused loss of gene expression from one generation to the next.

Five progeny rows from plants with resistance to hygromycin B in the R₃ generation also had resistance to Liberty herbicide in the R₄ generation. This showed that although Liberty resistance may not be expressed in early generations, it could be expressed in later generations. But some transgenic plants susceptible to both hygromycin B and Liberty herbicide in the R₃ generation did not express resistance to either compound in the R₄ generation.

3.3.5.3 Bacterial Panicle Blight Tests

All non-transgenic plants had 60% or greater BPB (Figure 3.17). Transgenic plants had different levels of diseased florets. More than 50% of the transgenic plants also had the 60% or greater BPB diseased florets (Figure 3.17). The resistance did not segregate as described by Shao (2003), but this did not necessarily mean that the thionin gene was not present in susceptible plants. Gene expression is often very complicated and can be affected by gene silencing.



Figure 3.15. Transgenic plants with Liberty herbicide resistance reaction in each row.



Figure 3.16. Transgenic plants with Liberty susceptible reaction in each row.

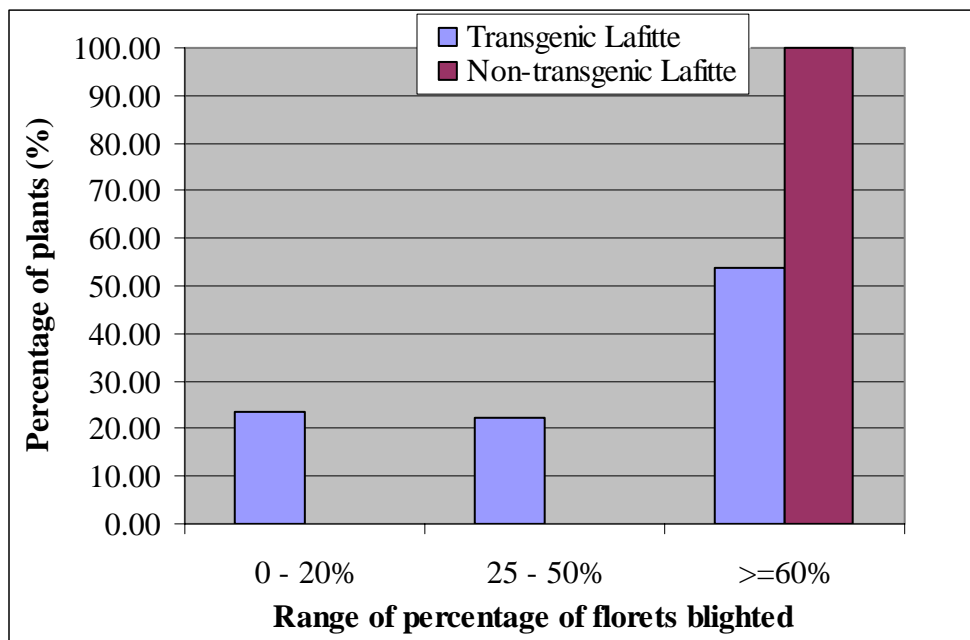


Figure 3.17. The range of the percentage of blighted florets among transgenic and non-transgenic R₄ Lafitte rice plants inoculated with *B. glumae* in a field test.

Inoculated non-transgenic Lafitte plants, with a percentage of florets blighted equal to or greater than 60%, had a significantly lower percentage of filled kernels than inoculated transgenic Lafitte plants with the percentage of florets blighted of 0 to 20% and 25 to 50% (Table 3.2). Transgenic plants with a percentage of florets blighted equal to or greater than 60% were not significantly differ in percentage of filled kernels from the non-transgenic control plants.. Also, the panicle weight of inoculated transgenic plants in the 0 to 20% of florets blighted range was significantly higher than inoculated non-transgenic plants, but panicle weights of transgenic plants in the 25 to 50% and >=60% florets blighted range were not significantly different in panicle weight from the non-transgenic control plants (Table 3.3).

The percentage of filled kernels on inoculated panicles was significantly correlated with panicle weight, with a correlation coefficient of 0.50 (p-value <0.0001). The percentage of filled kernels was significantly negatively correlated with the percentage of florets blighted, with a

correlation coefficient of -0.57 (p-value <0.0001). The panicle weight was also significantly negatively correlated with the percentage of florets blighted with a correlation coefficient of -0.45377 (p-value <0.0001).

Table 3.2. Multiple comparison of the percentage of filled kernels in each percentage of blighted florets range between inoculated panicles of transgenic plants and non-transgenic plants.

Percentage of blighted florets	Number of plants	Percentage of filled kernels (%)	Standard error
0-20%	35	58.83a ^x	2.7129
25-50%	34	40.94a	2.8335
>=60%	80	31.92	1.7867
Non-transgenic Lafitte	10	26.57	5.1473

^x significant at 0.05 level compared with non-transgenic Lafitte according to Dunnett's t Tests for percentage of filled kernels.

NOTE: This test controls the Type I experimentwise error for comparisons of all groups against a control.

Table 3.3. Multiple comparison of panicle weight in each percentage of blighted florets range for inoculated panicles of transgenic Lafitte R₄ plants compared with non-transgenic plants.

Percentage of florets blighted	Number of plants	Means of head weight (g)	Standard error
0-20%	35	2.31a	0.1010
25-50%	34	2.01	0.1055
>=60%	80	1.70	0.0665
Non-transgenic Lafitte	10	1.59	0.1917

^x significant at 0.05 level compared with non-transgenic Lafitte according to Dunnett's t Tests for panicle weight.

NOTE: This test controls the Type I experimentwise error for comparisons of all groups against a control.

Non-inoculated control plants did not fill 100% of their kernels under field conditions, therefore inoculated plants should be compared with non-inoculated plants to factor out failure to fill kernels due to environmental factors. The number of filled kernels on inoculated panicles and non-inoculated panicles for each transgenic plant were also investigated. Statistical analysis showed that panicles of transgenic plants with less than 20% of florets blighted had significantly more filled kernels than non-inoculated panicles (Table 3.4). In the 2004 season, stink bug populations were very high in the test field. After the crossing bags were placed onto partially emerged panicles, the panicles may have been protected from stink bug damage. Inoculated transgenic panicles with 25-50% of florets blighted had less filled kernels, but were not significantly different from non-inoculated panicles. Inoculated panicles with greater than 60% of florets blighted had significant less filled kernels than non-inoculated panicles (Table 3.4).

Inoculated transgenic plants with equal to or greater than 60% of florets blighted had a mean of 41 filled kernels/ panicle, which was similar to the 38 filled kernels/ panicle of inoculated non-transgenic plants. Non-inoculated transgenic plants in different percentage of florets blighted groups had similar numbers of filled kernels, but non-transgenic plants had fewer filled kernels than transgenic plants.

There were 35 transgenic plants with the percentage of florets blighted up to 20%, among them there were five plants (L-2-2, L-2-3, L-2-4, L-2-5 and L-9-1) resistant to Liberty in the R_3 generation, there were three plants (H-10-5, H-8-1 and H-6-1) resistant to hygromycin B in the R_3 generation, and there was one plant (HL-5-6) resistant to both hygromycin B and Liberty in the R_3 generation. Therefore, transgenic plants with resistance to Liberty or hygromycin B, but without resistance to seedling inoculated *B. glumae* in the R_3 generation were resistant to panicle inoculated *B. glumae* in the R_4 generation in the field test.

Table 3.4. Comparison of numbers of filled kernels between inoculated and non-inoculated panicles for transgenic Lafitte R₄ plants and non-transgenic plants in each percentage of florets blighted group.

Rating group	Percentage of blighted florets	Mean number of kernels on inoculated panicles	Means number of kernels on non-inoculated panicles	t-value	P-value
1	0-20%	79a ^x	63	2.7596	0.0091
2	25-50%	54	62	-1.0477	0.3026
3	>=60%	41a	52	-5.9436	<0.0001
Non-transgenic Lafitte (>=60%)		38a	45	-3.4775	0.0070

^x significantly different at the 0.01 level of mean filled kernels comparing inoculated panicle with non-inoculated panicle based on the t-test.

3.3.5.4 Demonstration of the Presence of the *bar* and Thionin Genes in Transformed Resistant Plants Using PCR

Leaf samples from seven transgenic Lafitte plants and a non-transgenic Lafitte control plant were tested using PCR. The results indicated that five transgenic plants had the *bar* gene (Figure 3.18), but only the transgenic plants in lanes 8, 9 and 10 were resistant to Liberty herbicide. Transgenic Lafitte plants in lanes 4 and 5 had the *bar* gene but did not have resistance to Liberty herbicide, which indicated that *bar* gene was silenced in these two plants. Transgenic plants in lanes 6 and 7 did not have the *bar* gene. This may be because after several generations of selfing, the *bar* gene was lost through segregation. Four samples from transgenic plants had the thionin gene, although lane 4 had a weak band it was visible (Figure 3.19). The BPB levels on the four plants, shown in lanes 4, 8, 9 and 10 were 10%, 40%, 20% and 30%, respectively. Disease resistance at different levels indicated that the expression of the thionin gene was different in each plant. One sample, shown in lane 5, had 5% of florets blighted, but the thionin gene was not amplified.

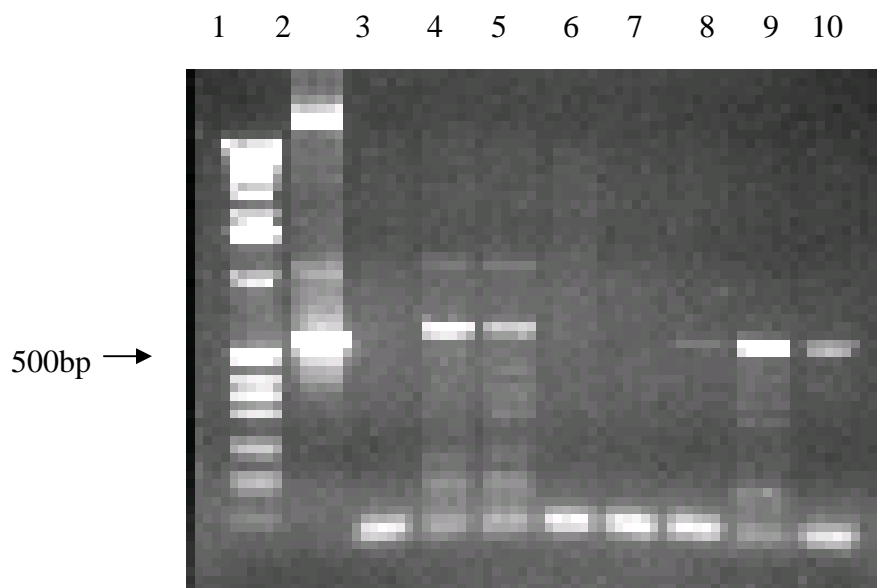


Figure 3.18. PCR analysis for *bar* gene on transgenic and non-transgenic Lafitte plants. Lane 1: 1kb molecular marker; lane 2: positive control; lane 3: non-transgenic plants; lanes 4 to 7 represent transgenic plants without resistance to Liberty herbicide; lanes 8 to 10 refer to transgenic plants with resistance to Liberty herbicide.

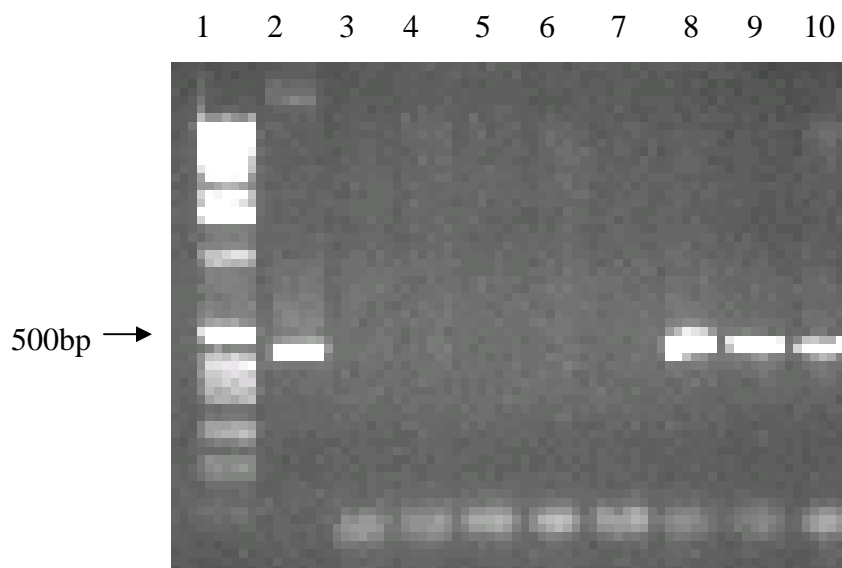


Figure 3.19. PCR analysis for the thionin gene in transgenic and non-transgenic Lafitte plants. Lane 1: 1kb molecular marker; lane 2: positive control; lane 3: non-transgenic plant; lane 4: a transgenic plant with 10% florets blighted; lane 5: a transgenic plant with 5% florets blighted; lane 6: a transgenic plant with 70% of florets blighted; lane 7: a transgenic plant with 40% of florets blighted; lanes 8 to 10: transgenic plants with 40%, 20% and 30% of florets blighted.

3.4 DISCUSSION

All five regenerated R₀ plants had a high level of resistance to Liberty and hygromycin B when compared with non-transgenic control plants. There was not a significant difference in the amount of BLB between transgenic and non-transgenic plants based on lesion lengths, which indicates Lafitte may have natural resistance genes.

Among the R₂ plants, BPB resistance and susceptibility did not follow normal inheritance rules, indicating that the expression of the thionin gene is much more complicated than expected.

Theoretically, all transgenic plants should be resistant to hygromycin B as both the thionin and *bar* genes had the *hpt* gene as the selective marker, and transgenic plants were selected on a medium with hygromycin B. However, after transgenic plants were transferred to the greenhouse and field, the *hpt* gene was not necessarily expressed and the selection pressure for maintaining the hygromycin resistance gene was no longer present. Therefore, some transgenic plants were susceptible to hygromycin B in greenhouse test and field tests.

R₃ transgenic plants tested with *X. oryzae* did show significant differences in resistance when compared to non-transgenic plants. This further suggested that Lafitte may have natural resistance to BLB.

Field testing for hygromycin B resistance in R₄ Lafitte plants showed segregation ratios that were not typical for a single dominant gene. This suggested that expression of the *hpt* gene may be affected by environmental conditions and by factors such as gene silencing or insertion location in the plants chromosomes. Segregation of resistance to Liberty herbicide also was not normal for a single dominant gene, probably for the same reasons. Gene silencing among the transgenic plants in this study was clearly demonstrated.

Phenotypic expression of the thionin gene was more complicated. Disease resistance at different levels suggested that expression of the thionin gene was different in different transgenic plants. One transgenic plant had 5% disease rating but the thionin gene was not amplified in the PCR products. BPB development is known to be affected by environmental factors, such as high temperatures favoring disease development, or by late inoculations. This may have happened with this false positive resistant plant. The inoculation stage is critical as the plant is at the stage of maximum susceptibility for only 2-3 days.

The higher the percentage florets infected, the lower the percentage of filled kernels and panicle weight, indicating that the system for rating disease was successful. Assessment of disease levels was correct and did not contribute to the abnormal segregation levels.

Some transgenic plants without resistance to seedling inoculation with *B. glumae* in the R₃ generation in greenhouse tests showed resistance to panicle inoculation with *B. glumae* in the R₄ generation in field tests. This suggests that seedling inoculation may differ from panicle inoculation, and the gene may not express the same way in different generations or under different environmental conditions.

Using Liberty as the selection marker for disease resistance in segregating populations is possible, but the resistance to BPB must be closely linked to resistance to Liberty herbicide resistance and the *bar* gene must be expressing. To quantify gene expression levels, real-time PCR should be conducted in the future.

This research has demonstrated that rice can be co-transformed with useful genes, with all of the genes expressed in some of the transgenic plants. Further research needs to be conducted to determine if the genes are linked in at least some of the plants.

CHAPTER 4

SUMMARY AND FUTURE RESEARCH

4.1 SUMMARY

Rice sheath blight is a major disease of rice worldwide and is the most important disease on rice in Louisiana. Since complete resistance has not been identified, breeders have been trying to develop resistant varieties based on partial resistance (Rush et al. 1984, 1995, 1996). This has worked to some degree, but higher levels of resistance are needed. Molecular plant biology has been introduced to the area of plant pathology and some PR protein genes have been identified and cloned. The transfer of PR genes and expression of gene products that are directly toxic, or reduce the growth of pathogens, is a major new area of research in rice breeding and plant pathology. Co-transformation of PR genes to inhibit fungal diseases has been successfully used in rice, maize, tobacco and tomato (Tang et al., 1999; Lyznik et al., 1989; Chen et al., 1998; Jongedijk et al., 1995). Gelvin (1998) illustrated the possibility of inserting more than one gene into one position on a chromosome through co-transformation with the target genes in different plasmids.

The chitinase gene from bean, the β -1, 3-glucanase gene from tobacco, and the *bar* gene were successfully co-transferred to calli derived from Taipei 309 using the *hpt* gene as the selective marker. Transgenic plants were regenerated and tested for resistance to hygromycin B, Liberty herbicide, and *R. solani*.

In both field and greenhouse tests, 200ppm ai of hygromycin B was used to test for resistance to hygromycin B using the cut leaf/dip method (Shao, 2003). The extended lesions and necrotic lesions on tested leaf blades were measured. Strongly resistant plants did not show any lesions. Moderate resistance could not be readily detected with this method. In greenhouse tests,

the differences in lengths of necrotic lesions produced by Liberty concentrations of 750ppm and 363ppm, and the differences in extended lesions produced by Liberty concentrations of 750ppm and 363ppm were not significantly different. As the environmental conditions in the greenhouse were less favorable for disease development than in the field, plants with low levels of resistance were not readily distinguished from plants with a high level of resistance.

Both 363ppm and 750ppm ai of Liberty herbicide were used to detect resistance in field and greenhouse tests. Highly resistant plants were easily identified as they showed no lesions. Resistance levels varied based on lesion length and higher concentration of Liberty produced longer lesions on moderately resistant plants. Lesion development under field conditions was more severe than in greenhouse tests. In the field, plants with low levels of resistance had significantly longer lesions when tested with 750ppm ai Liberty. But in the greenhouse, the two Liberty concentrations did not produce lesions that were significantly different.

Based on the field and greenhouse inoculation of transgenic plants with *R. solani*, the ratio of lesion length/plant height and lesion length were not significantly different for detecting SB resistant plants. Using both the disease ratio and lesion lengths to group the transgenic plants into different resistance groups based on cluster analysis worked well. Both the disease ratio and lesion length were highly correlated with the 0-9 SB disease rating at maturity in field tests.

In the field, plant height of transgenic Taipei 309 plants was not significant different from that of non-transgenic Taipei 309 plants. But in greenhouse tests, plant height differences were significant. Non-transgenic Taipei 309 plants were not significantly different from susceptible Cocodrie control plants for disease resistance. This was probably because the environment in the greenhouse was unfavorable compared to the field, so that plant height and disease development were significantly different from the field.

Two transgenic plants (15-7 and 33-4) were found with the β -1, 3-glucanase and chitinase transgenes, but they did not show high levels of resistance to SB indicating gene silencing. Plants with high level resistance, but without the transgenes were not found, which indicated that the resistance observed was from transgenes.

The *bar*, β -1, 3-glucanase, and chitinase genes were all found in the tested transgenic plants (except 9-2 without the *bar* gene) as proved by PCR and southern blot, but high expression for all the transgenes not observed. Most sheath blight resistant plants were also resistant to hygromycin B. Using hygromycin B as a marker for selecting disease resistant calli and plants in culture may make hygromycin B more reliable for selecting disease resistant plants in the field than Liberty herbicide. Liberty herbicide could only be used for selecting SB resistant plants in the field if the *bar* and SB resistance genes were linked.

Bacterial panicle blight can cause yield loss up to 40% (Shahjahan et al., 2000), and no pesticides are been available to control this disease in the United States (Shahjahan et al, 2001). This study used the co-transformation of thionin and *bar* genes to Lafitte rice to generate plants with resistance to both BPB and Liberty herbicide.

The thionin gene from barley, and the *bar* gene were successfully co-transferred to calli derived from Lafitte rice using the *hpt* gene as the selective marker. Transgenic plants were screened against hygromycin B, Liberty herbicide, *X. oryzae*, and *B. glumae* in different generations.

The five transgenic R₀ plants obtained were screened with hygromycin B and Liberty and all five transgenic plants were resistant to both compounds. PCR tests showed that all three of the target transgenes were in the five transgenic plants.

Panicles of R₂ plants inoculated in field tests showed moderate BPB resistance and analysis by RT-PCR showed that the thionin gene was being expressed in some of the plants. Only one transgenic plant with a high level of resistance (5% of kernels infected after inoculation) was observed, but grain yield was significantly higher in moderately resistant transformed plants than that produced by susceptible non-transgenic plants. Most transgenic plants in the R₃ generation with resistance to hygromycin B and Liberty herbicide also had resistant progeny in the R₄ generation. Some transgenic plants with resistance to either hygromycin B or Liberty herbicide in the R₃ generation showed resistance to both hygromycin and Liberty herbicide in the R₄ generation. A few transgenic plants with resistance to one or both hygromycin B and Liberty in the R₃ generation became susceptible in the R₄ generation suggesting that gene silencing occurred.

Transgenic and non-transgenic Lafitte plants both had similar reactions to *X. oryzae* in the R₀ and R₃ generations, which suggested that Lafitte may have natural resistance to BLB.

Transgenic plants with 0 to 20% florets blighted had significantly less disease and higher grain yield than non-transgenic plants, which indicates resistance. Some transgenic plants resistant to seedling inoculation with *B. glumae* in the R₃ generation did not have resistance to panicle inoculation in the R₄ generation. This may be because the gene was not expressed or was not expressed very strongly, as the thionin gene was detected by PCR in these plants.

Hygromycin B or Liberty herbicide could be used as a selective marker for selecting panicle blight resistant plants in segregating F₂ populations, but some disease resistant plants may be lost in later generations due to gene silencing.

4.2 FUTURE RESEARCH

From southern blot results, there were 3-4 copies each for the β -1, 3-glucanase and chitinase genes in each tested transgenic plant. It has been shown that copy numbers were correlated with the amount of DNA used (Chen et al., 1998), but the optimum amount of DNA used for transformation to obtain a single copy of each gene has not been established. Also the signal for each plant did not have the same intensity, but the reason was not clear.

Some resistant transgenic Taipei 309 plants were crossed with the susceptible variety Cocodrie last year by Dr. Q.M.Shao. More studies are needed to determine whether the SB resistance levels observed in Taipei 309 can be transferred by crossing to a susceptible variety like Cocodrie, how the resistance will segregate in further generations, and if the segregation will follow a 3:1 Mendelian ratio as described by Chen (1998).

Transgenic Lafitte plants were tested through the R_4 generation. Some Liberty resistant plants in the R_3 generation were not resistant to hygromycin B, but some progeny became resistant to hygromycin B in the R_4 generation. Also some R_3 plants resistant to hygromycin B were not resistant to Liberty, but progeny became resistant to Liberty in the R_4 generation. It is not clear how the resistance was recovered.

Transgenic Taipei 309 and Lafitte plants had different levels of resistance to disease based on transgenes. Using real-time PCR to quantify the gene expression is needed.

The transgenic Taipei 309 and Lafitte plants developed in these studies need to be tested for resistance to other fungal and bacterial diseases to determine the spectrum of resistance provided by the PR genes used.

In these studies rice was successfully transformed with the PR genes for chitinase,

β -1, 3-glucanase, and thionin production. The genes were demonstrated in transformed plants by PCR and southern blot tests. Many of the same plants were also transformed with the genes for resistance to the toxic compounds hygromycin B and Liberty herbicide. It appears that co-transformation will provide a mechanism to link transgenes in transformed plants in such a way that useful genes for disease resistance can be identified in segregating populations without resorting to sometimes difficult and expensive disease screening tests.

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